A STUDY OF EXPERIMENTAL HETEROTOPIC OSSIFICATION INDUCED BY URINARY BLADDER EPITHELIUM

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VOLUME I

TEXT and TABLES

I declare that the work reported has not been submitted for another degree, is my own, and that the thesis based upon this work was composed by me.

"When you can measure what you are speaking about and express it in numbers, you know something about it; but when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of science, whatever the matter may be."

LORD KELVIN.

I N D E X

VOLUME I - TEXT and TABLES — Page

VOLUME II - FIGURES

ABSTRACT

Heterotopic ossification can be induced experimentally following the implantation of certain types of epithelium, or devitalized hard tissues. In this thesis autotransplants of guinea pig urinary bladder epithelium to the anterior abdominal wall were examined. The animals were killed at intervals of up to six months, and the transplant area studied with the light and electron microscopes. All histological material was serially sectioned and measured with a semi-automatic image analysis system to assess accurately the development of cyst and bone. Maps of the cyst wall were drawn from section tracings and all results were analysed statistically.

Fibrin clots formed between transplant mucosa and site musculature shortly after operation. At Day 2 the epithelium of the transplant edge began to proliferate and extended between the clot and transplant mucosa to form a cyst by Day 8. Meanwhile the clot was replaced by fibrous tissue, within which at Day 10 the first osteogenic foci were found. During the next seven days matrix vesicles could be observed at the surface of developing bone. From Day 17 onwards osteoclasts were present.

Occasionally after Day 17, beneath the original transplanted epithelium, small osteogenic areas were observed.

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The cyst contained small tetrahedral crystals in some cases, between Day 10 and Day 100. Within the epithelium of the cyst lining maturation changes were observed from Day 9 and a typical urinary bladder epithelium had developed by Day 40. Several forms of epithelial aberrancy were found; outgrowths beneath the cyst lining (some associated with bone formation), and three forms of hyperplasia. It appeared that at about Day 40, areas of epithelium overlying the bone were shed and yet only beneath such areas did bone formation continue.

CHAPTER 1

LITERATURE

GENERAL INTRODUCTION

Heterotopic ossification, the development of bone in tissue other than skeletal, is a well-documented occurrence in mammalia. This type of bone formation may occur in any organ of the body. Most examples are found within pre-existing areas of metastatic or dystrophic calcification. The walls of sclerotic blood vessels in man are a good example.

The most dramatic example of heterotopic ossification is myositis ossificans progressiva. This extremely rare condition, usually found in children, is characterised by a progressive ossification of muscles, tendons, ligaments, fasciae and aponeuroses. After some years the condition usually becomes terminal due to the involvement of the heart and respiratory muscles. An apparently similar condition, myositis ossificans circumscripta, is seen following traumatic muscle injury, usually in the adult. Here the ossification is localized and non-progressive, apparently developing in the organizing tissues of the muscle haematoma. Heterotopic ossification is also frequently found in muscles paralysed as a result of spinal injury. This bone formation may be so extensive that it severely restricts movement at the joints concerned, usually the hip and knee joints.

Experimentally /

Experimentally heterotopic ossification has been produced by many methods. These can be arranged into three groups.

(a) Trauma to tissues and injection of skeletal extracts. - Table 1

(b) Implantation of devitalized skeletal tissues. - Table 2

(c) Implantation of epithelial tissues. - Table 3

TABLE 1

TRAUMA TO TISSUES AND INJECTION OF SKELETAL EXTRACTS

Trauma to Muscular Sites

TABLE 1

TRAUMA TO TISSUES AND INJECTION OF SKELETAL EXTRACTS

Trauma to Muscular Sites

Author	Animal	Age	Stimulus	Site	No. of animals	No. in which bone formed	Notes
HAGA & FUJIMURA (1904)	Rabbit	N.S.	Severe hammer blows	Thigh muscle	1	1	Bone found 28 days after stimulus
	Rabbit	N.S.	Repeated less severe blows	Thigh muscle	1	0	-
ASAMI, G. & DOCK, W. (1920)	Rabbit	N.S.	Calcium chloride, carbonate, sodium phosphate mixture	Leg muscle or beneath skin	5	0	No bone found 50 days after injection
SEVERI, R. (1933)	Rabbit	N.S.	Quinine (repeated)	Thigh muscle	15	3	-
			Other Tissue Sites				
HARVEY, W.H. (1907)	Rabbit	Adult	3% silver nitrate or 2% cupric sulphate solution	Abdominal Aorta	5	5	Bone in calcified media all animals after 110 days
IMAI, N. (1930)	Rabbit	Adult	Formic acid	Vitreous body of eye	26	12	Bone or cart. found in 12 cases
ENGLESTAD, R.B. (1934)	Rabbit	Young Adult	Sub-lethal x-radiation	Lungs	21	10	Fibrosis was followed by bone or cartilage formation after 60 days
	Rabbit	Young Adult	Lethal x-radiation	Lungs	7	5	Fibrosis was followed by bone or cartilage formation after 60 days

N.S. = Not stated.

TABLE 1 (contd.)

TRAUMA TO TISSUES AND INJECTION OF SKELETAL EXTRACTS

Injections of Skeletal Extracts

TABLE 1 (contd.)

TRAUMA TO TISSUES AND INJECTION OF SKELETAL EXTRACTS

Injections of Skeletal Extracts

Author	Animal	Age	Injected	Site	No. of animals	No. in which bone formed	Notes
LEVANDER, G. (1938)	Rabbit	N.S.	Alcoholic extract of bone and fracture callus (autogenous)	Rectus femoris muscle	60	14	Bone or cartilage found in 14 animals
	Rabbit	N.S.	40% alcohol	Rectus femoris muscle	60	0	
ANNERSTEN, S. (1940)	Rabbit	N.S.	Alcoholic extract of juvenile homogenous bone	Rectus femoris muscle	8	4	Bone found after 19 days
	Rabbit	N.S.	Alcoholic extract of juvenile heterogenous bone	Rectus femoris muscle	9	1	Bone found after 80 days
HEINEN, J.H. et al (1949)	Rabbit	Young	Alcoholic extract of bone	Triceps and quadriceps muscles	12	3	Bone or cartilage found in three animals
	Rabbit	Young	Alcohol	Triceps and quadriceps muscles	11	2	Bone or cartilage found in three animals
BRIDGES, J.B. & PRITCHARD, J.J. (1958)	Rabbit	Young Adult	40% alcohol	Rectus femoris muscle	8	0	No bone after 35 days
	Rabbit	Young Adult	Methylated Spirit	Rectus femoris muscle	12	1	Bone found in one case after 35 days
			Implantation of alcohol fixed muscle				
BRIDGES, J.B. & PRITCHARD, J.J. (1958)	Rabbit	Young Adult	Homogenous skeletal muscle	Beneath kidney capsule	11	11	Bone or cartilage formation
	Rabbit	Young Adult	Homogenous smooth muscle	Beneath kidney capsule	8	7	Bone or cartilage formation
	Rabbit	Young Adult	Homogenous cardiac muscle	Beneath kidney capsule	5	4	Bone or cartilage formation

TABLE 1

A wide diversity of results can be found in the experimental studies of this phenomenon, and it is difficult to see any common factor, except tissue damage as a result of the experimental procedures. It would appear from the variety of irritant stimuli which resulted in bone formation, that the calcium salts acted as non-specific irritants. The demonstration of bone formation following implants of alcohol devitalized muscle (Bridges & Pritchard, 1958) suggests that the alcoholic extracts and also possibly alcohol itself acted simply as non-specific irritants. Only in the rabbit have alcoholic extracts of skeletal tissues been shown to be osteoinductive (Bridges, 1959).

The probable explanation of these results is that traumatised rabbit muscle liberates a bone inductor which then stimulates bone formation in the young granulation tissue which develops at the site of injury.

TABLE 2

EXPERIMENTS USING DEVITALIZED SKELETAL TISSUES

TABLE 2

EXPERIMENTS USING DEVITALIZED SKELETAL TISSUES

Author	Animal	Age	Implant	Site	No. of animals	No. in which bone formed	Notes
BARTH, A. (1893)	Cat	N.S.	Ashed bone	Peritoneal cavity	1	1	Bone found at 42 days
WÜRM (1930)	Rabbit	Adult	Ashed bone	Anterior abdominal wall	N.S.	0	These results found between 90-120 days
	Rabbit	Young	Ashed bone	Anterior abdominal wall	10	6	These results found between 90-120 days
KIMBALL, R.M. (1949)	Rabbit	N.S.	Boiled bone (H)	Anterior chamber of eye	5	1	-
URIST, M.R. & MACLEAN, F.C. (1952)	Rat	N.S.	Boiled fracture callus (A)	Anterior chamber of eye	3	2	These results found at 36 days
	Rat	N.S.	Frozen fracture callus (A)	Anterior chamber of eye	3	1	These results found at 36 days
BRIDGES, J.B. & PRITCHARD, J.J. (1958)	Rabbit	Young Adult	Fracture callus (F.H.)	Beneath kidney capsule	19	13	Bone present from 30 days
	Rabbit	Young Adult	Xiphisternal cartilage (F.H.)	Beneath kidney capsule	4	0	-
	Rabbit	Young Adult	Epiphyseal cartilage (F.H.)	Beneath kidney capsule	12	10	Bone present from 30 days
	Rabbit	Young Adult	Ear cartilage (F.H.)	Beneath kidney capsule	6	0	-
URIST, M.R. (1965)	Rat	N.S.	Bone (D.H.)	Anterior abdominal wall musculature	N.S.	N.S.	Bone found in 90% of cases
YEOMAN, J.D. & URIST, M.R. (1967)	Rabbit	Young Adult	Bone (D)	Anterior abdominal wall musculature	N.S.	N.S.	Authors state, "Nearly always positive for bone formation."
	Rabbit	Young Adult	Dentine (D)	Anterior abdominal wall musculature	N.S.	N.S.	-
NOGAMI, H. & URIST, M.R. (1975)	Rat	Adult	Bone matrix gelatin (within diffusion chamber)	Anterior abdominal wall musculature	65	52	-

A = Autogenous D = Decalcified in cold 0.6N HCL F = Alcohol fixed H = Homogenous N.S. = Not stated by author

TABLE 2

The results of the earlier experiments would suggest that ossifying or ossified tissues, devitalised by various methods, were able to induce bone formation in soft tissues. However, most of the early experiments were conducted in the rabbit, where heterotopic ossification occurs very readily.

Urist (1965) reported a series of experiments in which he used bone, decalcified in cold 0·6 N hydrochloric acid, as a stimulus. He found that homogenous bone treated in this manner (whether previously fixed or not) was osteoinductive in 90% of cases when implanted into rats, mice, guinea pigs, or rabbits. If heterogenous decalcified bone was used, bone formation occurred in only 60% of cases. The new bone formed from four weeks after operation, and was always restricted to within the original implant. Yeoman & Urist (1967) demonstrated that dentine, treated in a similar manner was also osteoinductive.

Thus the presence of an osteoinductive principle in bone and dentine matrix has been demonstrated. The humoral nature of this principle was shown by Nogami & Urist (1975).

TABLE 3

EXPERIMENTS USING EPITHELIAL TISSUES

Transplants of non-urinary epithelium

TABLE 3

EXPERIMENTS USING EPITHELIAL TISSUES

Transplants of non-urinary epithelium

Author	Animal	Age	Stimulus	Site	No. of animals	No. in which bone formed	Notes	Onset of ossification
NAKAMOTO, Y. (1927)	Guinea Pig	N.S.	Gall bladder wall (A)	Anterior abdominal wall	18	N.S.	"Almost all animals showed bone formation after 14 days"	14 days
HUGGINS, C.B. & SAMMETT, (1933)	Guinea Pig	N.S.	Gall bladder wall (A)	Anterior abdominal wall	12	9		14 days
	Dog	N.S.	Gall bladder wall (A)	Anterior abdominal wall	3	2	Four animals killed prior to 41 days - no bone formation	41 days
	Dog	N.S.	Fascia (A)	Gall bladder defect	4	4		N.S.
ANDERSON, H.C. et al (1964)	Mouse (cortisone treated)	Young	Cultured human amnion cells	Thigh muscle	17	17		12 days
HUGGINS, C.B. (1969)	Guinea Pig	Young	Seminal vesicle (A)	Anterior abdominal wall	14	8		22 days

A = Autogenous H = Homogenous N.S. = Not stated by author

TABLE 3 (contd.)

EXPERIMENTS USING EPITHELIAL TISSUES

Trauma to urinary tract

TABLE 3 (contd.)

EXPERIMENTS USING EPITHELIAL TISSUES

Trauma to urinary tract

Author	Animal	Age	Stimulus	Site	No. of animals	No. in which bone formed	Notes	Onset of ossification
WEINLAND, D.F. (1859)	North American Mink	N.S.	Infestation with nematode	Kidney	N.S.	N.S.	Bone found in kidneys of which medulla was destroyed by nematode	N.S.
PEARCE, R.M. (1909)	Dog	N.S.	Partial excision	Kidney including Pelvis	14	6	Bone found in scar tissue	22 days
HUGGINS, C.B. (1931)	Dog	N.S.	Diathermy	Kidney Pelvis	3	2	Bone found in granulation tissue beneath urinary epithelium	40 days
	Dog	N.S.	95% Phenol	Kidney Pelvis	N.S.	1		52 days
				Ligation of renal vessels				
SACERDOTTI, C. & FRATTIN, G. (1902)	Rabbit	Young Adult	Ligation of blood supply	Kidney	4	3	Animals killed between 74-85 days	N.S.
BRIDGES, J.B. (1958)	Rabbit	Adult	Ligation of blood supply	Kidney	9	9		40 days
	Rat	N.S.	Ligation of blood supply	Kidney	7	7		Between 40 and 70 days
	Guinea Pig	N.S.	Ligation of blood supply	Kidney	8	2	All kidneys became severely infected	Approx. 100 days

N.S. = Not stated by author

TABLE 3 (contd.)

EXPERIMENTS USING EPITHELIAL TISSUES

Experimental surgery of urinary tract

TABLE 3 (contd.)

EXPERIMENTS USING EPITHELIAL TISSUES

Experimental surgery of urinary tract

Author	Animal	Age	Stimulus	Site	No. of animals	No. in which bone formed	Notes	Onset of ossification
STRAUSS, A.A. (1914)	Dog	N.S.	Repair with fascial flap	Ureter	4	4	Fascia ossified at 180-210 days	N.S.
NEUHOF, H. (1917)	Dog	Varied	Repair with fascial patch	Bladder	14	14	Fascia ossified	17 days
PHEMISTER, D.B. (1923)	Dog	N.S.	Repair with fascial patch	Bladder	N.S.	N.S.	Bone formed in all cases	N.S.
	Rabbit	N.S.	Repair with fascial patch	Bladder	4	0	This result at 40 days	N.S.
	Sheep	N.S.	Repair with fascial patch	Bladder	1	0	This result at 50 days	N.S.
			Transplants of urinary epithelium					
HUGGINS, C.B. (1931)	Dog	N.S.	Transplant of bladder mucosa (A)	Anterior abdominal wall	44	44		19 days
	Rabbit	N.S.	Transplant of bladder mucosa (A)	Anterior abdominal wall	6	1	This bone found at 95 days	-
HUGGINS, C.B. et al (1936)	Guinea Pig	N.S.	Transplant of bladder mucosa (A)	Anterior abdominal wall	3	3		N.S.
	Rat	N.S.	Transplant of bladder mucosa (A)	Anterior abdominal wall	4	4		N.S.
JOHNSON, F.R. & McMINN, R.M.H. (1956)	Cat	Adult	Transplant of bladder mucosa (H)	Anterior abdominal wall	20	10		10 days
	Cat	Adult	Transplant of bladder mucosa (A)	Anterior abdominal wall	19	15		10 days
FRIEDENSTEIN, A.Y. (1968) *	Guinea Pig	N.S.	Transplant of bladder mucosa (A)	Anterior abdominal wall	N.S.	N.S.	Bone found in 90% of cases	10 days

* Review article citing earlier work published in Russian (Friedenstein, 1963).

TABLE 3

The first epithelium shown to be osteoinductive following transplantation, was that of the gall bladder (Nakamoto, 1927). Shortly afterwards, Huggins (1931) demonstrated that urinary bladder epithelium was also osteoinductive. Both epithelia induce bone formation in a high proportion of experiments but the onset was earlier following stimulation by urinary epithelium.

In 1964, Anderson et al, reported that when cultured human amnionic cells were injected into cortisone-treated mice, bone formed as a response, in practically every case. However, the bone formed endochondrally, unlike that found in response to implants of urinary or gall bladder epithelium, which formed intramembranously.

Seminal vesicle epithelium was also shown to be osteoinductive by Huggins (1969), and the formation was intramembranous in type.

HETEROTOPIC OSSIFICATION AND THE URINARY TRACT

An early example of heterotopic ossification in the urinary tract was reported by Weinland (1859) in a survey of parasitic worms found in humans. The nematode Strongylos gigas R. is occasionally found in human kidneys, but is a common parasite of the North American mink. Weinland described in this animal as many as six nematodes in one kidney, with total destruction of kidney parenchyma leaving only a thickened capsule. In almost every animal which had suffered destruction of the inner kidney by this parasite, a thin piece of bone was found in the remaining kidney tissue. The post-traumatic formation of bone in the urinary tract, was confirmed experimentally in the dog by Pearce (1909) and Huggins (1931).

In 1902 Sacerdotti and Frattin ligated the renal vessels in four rabbits. The animals were killed between three and four months later, and bone formation was found in the kidneys of three rabbits. The bone formed initially as a thin plate in the connective tissue, underlying the epithelium of the ureteric pelvis, close to the renal papilla. The bone was coarsely fibred and apparently formed by direct ossification of the connective tissue. This bone was later replaced by lamellar bone. Sacerdotti & Frattin interpreted the bone formation in these ischaemic rabbit kidneys, as a direct metaplasia of connective tissue and likened it to the /

the ossification seen in the developing cranial vault. These results have been confirmed by other workers e.g. Asami & Dock (1920) and Bridges (1958).

Bridges (1958) demonstrated that this process regularly follows ligation of the renal vessels in the rat and guinea pig, as well as the rabbit. All three animals show bone formation, initially very close to the urinary epithelium, which is never preceded by cartilage formation. Later after more extensive bone formation has occurred haemopoetic marrow is found between the bony trabeculae.

In 1914, Strauss observed bone formation in fascial flaps, used to repair experimental defects in dog's ureters. He reported that the fascial repair was converted to a bony tube, which strengthened the repair and kept the lumen patent. Neuhof (1917) reported a similar occurrence when he transplanted fascia into a defect of the urinary bladder wall in the dog. The fascial patch became ossified in all the animals approximately 17 days after operation. Phemister (1923), confirmed the results of Strauss and Neuhof, in the dog. Strauss, Neuhof & Phemister, all suspected that the acidic urine, found in the dog, stimulated this bone production. However, the same fascial repair of the bladder, in four rabbits and one sheep resulted in no ossification; these animals have alkaline urine.

The classical experimental work in this field (Huggins, 1931) /

(Huggins, 1931) first published in a preliminary report (Huggins, 1930) followed from the work of Strauss (1914), Neuhof (1917) and Phemister (1923). Huggins conducted a series of elegant experiments in dogs to elucidate the factors responsible for bone formation in the urinary tract.

Huggins repeated the experiments of Neuhof & Phemister, after prior diversion of all urine from the bladder. Three dogs, 55 days later, all showed a dense button of bone strictly limited to the fascial transplant, the surface of which was covered by urinary epithelium. These results suggested that the urine was not, after all, important.

Huggins then tried to establish the tissue responsible for the bone induction. He separated the bladder epithelium from the underlying muscle and connective tissue, then transplanted these two components of the bladder wall to different sites on the anterior abdominal wall. After 60 days a cyst lined by bladder epithelium was found at one site, but no evidence of the graft could be found at the other. The cyst of urinary epithelium was covered (on one side only) by living bone with haemopoietic marrow. This experiment was repeated 44 times with similar results. The author noted "vesical epithelium is particularly hardy and favourable for transplantation".

A study of the histogenesis then followed. Seven to nine days after operation "an epithelially-lined spheroid containing fluid had formed". The newly-formed epithelium /

epithelium of the cyst was considerably thinner than the transplanted epithelium, only one or two cells in thickness. By 14-16 days the newly-formed epithelium was more characteristic in appearance and difficult to differentiate from the grafted epithelium. At the same time the connective tissue surrounding part of the cyst exhibited oedema, "fibrillar" in type. This tissue was more eosinophilic and acellular, than other areas of connective tissue around the cyst, "presumably a pre-osseous stage". By 19 days the first spicules of bone could be seen adjacent to the newly-formed epithelium. After 19 days all cysts exhibited bone formation, which apparently reached a maximum about two months after operation.

The factors controlling the position of the bone formation were studied by variations of his earlier techniques. Huggins made two autogenous grafts of bladder epithelium to the anterior abdominal wall in one dog, with one graft positioned luminal surface towards the skin, and the other with its luminal surface towards the muscle. Both grafts gave rise to typical cyst formation, but in both cases the bone was only found close to the new growth of epithelium, in relation to skin in one case and muscle in the other.

The necessity for proliferating epithelium was also demonstrated in another experiment in which Huggins dissected the fibromuscular tissue from the bladder wall but left the epithelium intact. He filled the defect /

defect with a fascial graft. When examined up to 87 days later, no bone was found. Transplants of epithelium from all parts of the urinary tract were found to be competent inductors of bone formation. However the epithelium of the adrenal gland, gall bladder (see below) stomach, jejunum, colon and duramater, all failed to induce bone formation in the dog.

The response of different connective tissue sites to grafts of urinary bladder epithelium, was also examined. Bone formation occurred in the rectus muscle, rectus sheath, subcutaneous fatty tissue, fascia lata, sacrospinalis fascia and synovial cavity. However, transplants to the parenchyma of kidney, spleen, liver and lung, all failed to induce ossification. When the bladder wall was denuded of epithelium in situ and allowed to heal, no bone was formed. These results seem to suggest that two types of connective tissue can be recognized by their response to grafts of urinary epithelium.

Huggins also studied the response to grafts of urinary bladder epithelium in six rabbits. The grafts were placed in the rectus abdominis muscle, and bone was found in only one rabbit.

Huggins published several more papers in this field during the next five years. Two of these are of particular interest;

Huggins & Sammett (1933) reported that gall bladder epithelium was osteoinductive in the dog and guinea pig anterior /

anterior abdominal wall. The difference between the findings of this study and that of Huggins (1931) was probably due to the use of only two dogs in the earlier study, whereas seven were used in the later study, only two of which showed bone formation.

Huggins et al (1936) found that the osteogenic response to urinary bladder epithelium was very similar in the dog and guinea pig.

Huggins demonstrated:

1. Transplanted urinary epithelium from bladder, ureter, or renal pelvis, consistently causes bone formation in dogs and guinea pigs.

2. Urine is not necessary for bone formation.

3. Two types of connective tissue can be distinguished by their response to urinary epithelium grafts.

4. Bone formation rarely occurs in the rabbit as a result of urinary epithelial grafts.

5. The new proliferating epithelium and not the original bladder wall or original epithelium, is the inducing agent.

TABLE 4

RESPONSE OF DIFFERENT SPECIES TO GRAFTS OF URINARY EPITHELIUM

TABLE 4

RESPONSE OF DIFFERENT SPECIES TO GRAFTS OF URINARY EPITHELIUM

Author	Animal	Histocom-patability	No. of animals	No. in which bone formed	Notes	Onset of ossification
HUGGINS, C.B. (1931)	Dog	Autogenous	44	44		19 days
MARSHALL, V.F. & SPELLMAN, R.M. (1957)	Dog	Autogenous	38	38		
	Dog	Homogenous	7	0		
JOHNSON, F.R. & McMINN, R.M.H. (1956)	Cat	Autogenous	19	15		10 days
	Cat	Homogenous	20	10		10 days
HUGGINS, C.B. (1931)	Rabbit	Autogenous	6	1	Animal killed after 95 days	
HUGGINS, C.B. et al (1936)	Guinea Pig	Autogenous	3	3	Confirmed by Friedenstein, (1963)	11 days
FRIEDENSTEIN, A.Y.* (1956)	Guinea Pig	Homogenous	N.S.	N.S.	States results initially same as autogenous	11 days
HUGGINS, C.B. et al (1936)	Rat	Autogenous	4	4		N.S.
FRIEDENSTEIN, A.Y. & LALYKINA, K.S.* (1963)	Rat	Homogenous	N.S.	N.S.	State both autogenous and homogenous grafts in rat only form bone in 10% of cases	20 days
ZALESKI, M. & MOSKALEWSKI, S. (1963)	Mouse	Autogenous	20	2		Between 30 and 60 days

* published in Russian - referred to by Friedenstein (1968) in an English paper

N.S. Not stated by author

TABLE 4

The response to autogenous grafts of urinary bladder epithelium varied widely between species. The formation of bone was apparently most consistent in the dog, guinea pig and cat. (The results found by Huggins et al (1936) in the rat, are subject to question in light of the work of Friedenstein and Lalykina (1963)). These three species may also be ranked according to speed of onset of ossification. The cat and guinea pig both commenced bone formation about 10-11 days after operation, whereas in the dog, the first bone was seen 19-20 days after operation. The reliability of bone formation in the guinea pig was considerably greater than in the cat (90% plus as opposed to 75% success rate). The above facts taken together would point to the guinea pig as the animal of choice, an opinion supported by all recent workers in this field (Friedenstein, 1968; Huggins, 1969 and Menon et al, 1974).

The variation between species was also considerable if homogenous grafts of urinary bladder epithelium were used. In the dog, a complete absence of bone formation was noted, in the cat the response was below that for autogenous grafts, whereas in the guinea pig, homografts and autografts were equally successful in inducing bone formation.

Several workers, Cascio & Brucia (1951), Lorenzi & Batacchi (1951) and Zaleski et al (1963), have studied the /

the response to heterogenous grafts to urinary bladder epithelium. In all cases no bone was found and the graft was rapidly destroyed by the immune response. However, if the immune response was controlled by cortisone therapy, bone formation could be induced, (Wlodarski, et al 1970). The urinary bladder epithelium of the dog, guinea pig, and Syrian hamster, when grafted into cortisone-treated mice resulted in bone formation. The bone formed was preceded by cartilage formation, a quite different type of ossification from that seen following auto- or homotransplantation. The bone formation was similar to that seen following the grafting of heterogenic amnionic human cell lines into cortisone-treated mice (Anderson et al 1964). The reasons for this similarity are far from clear but it may have been related to the cortisone therapy itself, perhaps producing a relative ischaemia around the transplants due to the suppression of the inflammatory response.

STUDIES IN THE GUINEA PIG

Histology

The histogenesis of bone formation following bladder mucosa transplantation has been described by Friedenstein (1961), Makin (1962), Huggins (1969) and Menon *et al* (1974).

Friedenstein (1961) described the changes seen, following homotransplantation of bladder mucosa to the anterior abdominal wall. He reported that by Day 2 the basal layer of transplanted epithelium exhibited signs of growth. At first, only one cell in thickness, the new growth was usually two cells thick by Day 5. The other cell layers of the transplanted epithelium did not appear to contribute to the new growth. About Day 6, small cysts were seen and also strands of epithelium, penetrating deep into the granulation tissue. This newly formed epithelium was at first undifferentiated but later areas of differentation were found. The differentiated areas were seen towards the periphery of the transplant. Three distinct cell types in their respective layers developed, similar in appearance to normal bladder epithelium '*in situ*'. This differentiation was usually complete by Day 15. A basement membrane was observed beneath this epithelium from Day 11. The undifferentiated areas consisted of a homogeneous cell layer 2-3 cells deep, the cells of which appeared similar to those of the intermediate cell layer of normal bladder epithelium. /

epithelium. By Day 8 the basal cells of this epithelium were seen penetrating into the underlying connective tissue. These ingrowths were most marked at Day 10. The author was unable to observe any basement membrane beneath this undifferentiated epithelium during the period of the study. The tissue beneath the differentiated epithelium became oedematous by Day 10 (whereas that beneath the undifferentiated epithelium exhibited intense cellular proliferation (Friedenstein, 1968). At about Day 10, the first signs of bone formation were reported, close to the epithelial outgrowths. About Day 13 further osteogenic centres appeared in the cyst wall, close to the epithelium and apparently more active than the earlier centres. Bone was only observed beneath areas of undifferentiated epithelium.

The cysts acquired a "typical appearance" about Day 18. By Day 25 degeneration of the transplant could be seen and soon thereafter the bone in the cyst wall was resorbed.

Makin (1962) studied transplants of autogenous or homogenous bladder epithelium, to the erector spinae fascia.

He described the histogenesis in five phases -

Day 1-5: Regeneration - epithelial cells regenerated surrounded by an inflammatory reaction.

Day 5-10: Cyst formation - cyst cavities developed in the epithelial islands and enlarged.

Day 10-15: /

Day 10-15: Pericystic Hyaline Formation - intensely eosinophilic hyaline homogeneous material accumulated beneath the epithelium.

Day 15-25: Osteogenesis - osteoid, then bone formed in the hyaline areas, only beneath new growth of epithelium.

Day 25 onwards: Formed bone - the bone, initially woven in type, converted to lamellar bone, with active bone marrow. New bone formation ceased at about five weeks, but the bone persisted for at least six months.

Huggins (1969) autotransplanted the 'dome' of the bladder to the anterior abdominal wall. He reported that the transplanted epithelium grew rapidly by lateral extension, and formed cysts which were completely closed by Day 7. These cysts were lined by thick epithelium, on the transplant side, which tapered to a layer one cell thick on the opposite cyst wall.

Beneath the new growth of epithelium, "small dark curvaceous cells" were seen, which from Day 10 were associated with spicules of bone. By Day 12 a macroscopic, thin disc of bone had formed, surrounded by osteoblasts. From Day 21, "non-responsive fibroblasts" separated the epithelium from the bone. This surface of the bone then ceased to grow although growth was observed for some time on the other surfaces. A dense mass was formed which from Day 28 onwards was populated by red bone marrow.

Menon /

Menon et al (1974) followed closely the technique of Makin (1962) and their description of the histogenesis was essentially the same.

Histochemistry

Glycogen (as revealed by P.A.S. technique)

The areas of cyst wall epithelium which had differentiated into a typical three-layered form, showed a gradient of glycogen concentration, from the basal cells which contained very little to the superficial cells which contained large amounts (Friedenstein, 1961; Makin, 1962). A similar distribution of glycogen has been described for 'in situ"guinea pig bladder epithelium, (Kanczak, 1964; Friedenstein, 1961).

Friedenstein (1961) also reported that those areas of epithelium in the cyst wall which remained undifferentiated, contained similar amounts of glycogen in all layers. The osteocytes within the bone induced by this epithelium contained glycogen but none was observed in the osteoblasts.

Other P.A.S. positive material

Leowi (1954), Friedenstein (1961), Makin (1962) and Menon et al (1974) all reported that the newly-formed bone in the cyst wall was strongly P.A.S. positive. Newly-formed bone has been similarly reported to be strongly P.A.S. positive, by Pritchard (1952) in the rat foetus, and by Bonucci & Gheradi (1975) in medullary bone of the pigeon. The hyaline material described by Makin was also faintly P.A.S. positive.

Ultrastructure /

Ultrastructure

Makin (1962) examined the ultrastructure of the bone induced by guinea pig bladder epithelium and compared it with newly formed bone in the metaphysis of a rabbit's long bone. The only difference he found was a larger proportion of uncalcified matrix in the induced bone. He suggested that this was a result of very rapid osteogenesis which outstripped the local calcium supply.

Abdin & Friedenstein (1972) studied the ultrastructure of bladder mucosal transplants and the bone induced by them. The transplanted epithelium which did not induce bone formation contained only cells resembling those of the intermediate layer in '_in situ_' epithelium. These areas of epithelium were always demarcated by a basal lamina from the underlying tissue, whereas none was found beneath the areas of epithelium which induced bone formation.

At Day 12 the first osteoblasts were found in the connective tissue beneath inductive epithelial cells, showing characteristically highly organized endoplasmic reticulum and eccentrically placed nuclei. The first signs of calcification were noted as star-shaped clusters on the collagen fibres between the osteoblasts. The clusters enlarged and eventually enclosed the cells but a collagenous zone 0.5-1.5μm wide around the osteocytes remained uncalcified. No differences between the ultrastructure of the induced bone and normal skeletal bone were reported.

POTENTIAL CLINICAL APPLICATIONS /

POTENTIAL CLINICAL APPLICATIONS

Several workers have studied the ability of bladder mucosal transplants to improve the quality and speed of bone repair.

In 1932 Copher et al produced bilateral ulnar defects in dogs. One side was a control, and the other side received an autograft of bladder mucosa across the defect. The grafted side developed sound bony union associated with many cysts, but the control side showed non-union and atrophy of the bone ends. Aqueous, alcoholic, and chloroform extracts of bladder mucosa were not effective in promoting bony union. Copher (1938) continued this work by placing bladder mucosa across ulnar defects produced 14-17 weeks earlier, in which rarefaction of the bone ends and non-union had already occurred, but 15 weeks after grafting nine of twelve defects showed bone union. However, when all the periosteum was removed, before the placement of the bladder mucosa, five of eight defects were bridged about one year later by a tubular mass of calcified tissue but solid union did not occur. Eskelund & Plum (1950) found that bladder scrapings increased the rate of healing of femoral fractures in young rats, but aqueous extracts were ineffective.

Makin (1962) produced bilateral defects of 3-4mm in the radii of young guinea pigs. One side was a control, the other received an autograft of bladder mucosa. Ten days /

days after operation bone could be seen radiographically in the defect; bone production was more widespread and rapid than in transplants to fascial sites. Fifteen days post-operatively, massive new bone formation was seen uniting the severed bone ends, surrounding a well-defined cyst. At this stage the control side showed no evidence of union. Bone production did not spread but bone bridging the defects matured and six to 12 months post-operative the cyst disappeared, leaving a hard bony swelling.

Beresford & Hancox (1967) studied the repair of cranial vault defects in the guinea pig and rat (a site where bone repair is usually poor). They bridged the defects with bladder mucosa pieces, dispersed in poly-urethane sponge or deproteinized cancellous bovine bone. Both autogenous and homogenous transplants enhanced the repair of the defects, but the autogenous epithelium persisted and produced large cysts which expanded into the cranial cavity.

The rapid stable bony union achieved, the quality of bone produced, and the absorption of the cyst, which Makin demonstrated, caused him to suggest a clinical application for this technique. However, Beresford & Hancox did not find that the autogenous epithelium used in their study was absorbed and so they suggested that only homogenous transplants of urinary epithelium were potentially clinically useful in assisting bone repair.

In 1973 Barker & Litton reported a further clinical application /

application of bladder mucosa induced bone formation. They studied a method for the repair of tracheal defects in 31 dogs, using autogenous bladder epithelium initially supported by a plastic former. Although only about a third of the animals survived for more than two weeks, those that did showed good functional results with a rigid airway supported by circumferential bone growth of uniform thickness. The lumen was lined by a mixture of pseudostratified and urinary epithelium. The authors stated that no cysts were found.

CHAPTER 2

MATERIALS and METHODS

EXPERIMENTAL ANIMAL

The animal chosen was the guinea pig (Cavia porcellus). Transplantation was attempted in both sexes and because the surgery was considerably easier to perform in females it was decided to use this sex. Guinea pigs are weaned at 21 days (120-220g) and reach maximum size at about 28 weeks, with a weight of 900-1,000g. The age chosen for transplantation was 50-60 days (370-470g); at this stage the animals were of a convenient size and it was found, following a study of more than 40 females in the Anatomy Department Animal House, that their rate of growth would be almost constant during the early experimental period. The strain used was Albino Dunkin-Hartley guinea pigs supplied by the Centre for Laboratory Animals, The Bush, Milton Bridge, Penicuik.

Husbandry

The guinea pigs were housed in the Animal House of the Department of Anatomy, University of Edinburgh. The growth study had shown an initial weight loss during the first few days following arrival in the Department, but this loss was restored within 7-10 days of arrival, and was followed by a steady weight gain thereafter. As a result of this information, the stock animals, which arrived in pairs (usually litter-mates) were obtained three /

three weeks before operation to allow complete adjustment to their new surroundings.

The animals were fed a proprietory guinea pig diet with meadow hay supplement and fresh tap water. No fresh greens or ascorbic acid supplements were given and no evidence of ascorbic acid deficiency was seen. Lighting was natural daylight and the air temperature was maintained between 60° and 80°F.

After operation the animals were kept in individual cages, on the same regime. Each animal was marked to ensure accurate identification and weighed regularly throughout the experiment.

OPERATIVE PROCEDURES

Anaesthesia

A satisfactory method for anaesthetising guinea pigs was found only after the trial of several different techniques.

The first technique tested had been used successfully in the Anatomy Department for anaesthetising rats. The animal was placed in an ether jar until it became drowsy, lifted out, and then given an injection of Nembutal intraperitoneally (33mg. Nembutal/Kg body wt.). Approximately ten minutes later surgical anaesthesia was achieved and more ether was administered during surgery when required.

This method was not reliable when applied to guinea pigs. Two main problems were found.

(1) The level of anaesthesia was extremely variable.

(2) /

(2) Several animals died from ether poisoning and/ or inhalation of vomit, during attempts to compensate for poor Nembutal anaesthesia.

Starvation several hours before surgery was inneffective in reducing this problem. There was, however, considerable improvement when 2% lignocaine (containing 1:80,000 adrenaline) was injected subcutaneously into the operation sites. Local anaesthetics were not used routinely because the introduction of chemicals into the transplant site was felt to be undesirable and wound healing was much worse following their use.

Vetalar (Parke-Davis), a new anaesthetic agent was tested. It was said to provide good anaesthesia, while not abolishing the laryngeal reflex. After a short period of use, it was found that the intra-peritoneal dose for a guinea pig was very large and it was confirmed by Parke-Davis that guinea pigs required an exceedingly high dose. As no improvement over Nembutal was found in this study, its use was abandoned.

Inhalational anaesthesia was the last technique examined. Initially an oxygen/nitrous oxide mixture (1:1) with Halothane vapour was used. (Halothane is a widely used inhalational anaesthetic agent) but when it was found that Halothane with oxygen alone, gave very satisfactory results, the use of nitrous oxide was discontinued. Oxygen was given at a flow rate of 1.5-2.0 litres/min. passed through a Gardener temperature compensated /

compensated vapouriser. Four per cent Halothane/Oxygen was used for induction, reduced to 3½% for maintenance. During induction the animal was placed in a plastic box flushed with the gas mixture (Fig. 1) but was transferred to a head mask for maintenance of anaesthesia (Fig. 2). The gases were delivered through a small bore open circuit system, and the apparatus was used only in a well-ventilated room, to minimise any hazard from waste gases.

This last method was finally adopted because it was very reliable, giving stable anaesthesia, muscle relaxation, rapid recovery, and very few animals were lost during anaesthesia. It was used for all surgery, removal of sutures, and the termination of experiments reported in this study. Starvation before an anaesthetic was not found to be necessary.

Surgery

The operation sites were shaved under surgical anaesthesia. After covering the animal with sterile drapes the prepared areas were swabbed with disinfectant solution (Dettol).

Two incisions were made, each approximately 20mm in length. The first was placed midway between the xiphoid cartilage and the pubic symphysis; slightly left of midline. If necessary this position was altered slightly to avoid possible contact between mammary glandular tissue and the transplant. A pocket was then made, by separating the skin from the anterior abdominal wall musculature, to the right of this incision for a distance of 15-20mm.

The /

The second incision was positioned parallel to the midline, half-way between the left major nipple and the midline and extended upwards from the perineum to terminate at a line between the major nipples. The skin around the incision was dissected free from the underlying mammary tissue, which was peeled in turn from the muscle beneath it and reflected downwards. An incision was then made in the muscle, in line with the upper part of the skin wound, and continued forwards beneath the uncut skin for about 5mm. The length of this incision was about 10mm. In order to avoid damage to the large bowel, the muscle was raised with a pair of forceps before being cut carefully with scissors.

The bladder lay to the right of the muscle wound and usually was distended. It was brought into view by gentle pressure on the right side of the abdomen and by increasing pressure on either side of the wound the bladder was evaginated through it (Fig. 3). The bladder was ligatured with a loop of chromic catgut while the apex was held in forceps by an assistant, one of the ligature ends was used as a stay to prevent the return of the bladder, the other was cut off (Fig. 4). Using a pair of fine pointed scissors the distal portion of the bladder was then excised leaving a cuff of bladder wall 1-2mm distal to the ligature, and the excised part (transplant) was placed in the subcutaneous pocket beneath the upper wound, prepared earlier. Two or three single sutures were used to repair the bladder defect (Fig. 5), the stay removed /

removed and the bladder allowed to return to the abdominal cavity. Next the transplant was fixed to the floor of the subcutaneous pocket by four single sutures (Fig. 6); the epithelial surface of the transplant apposed to the anterior abdominal musculature. Securely tied single sutures were used to repair the lower incision muscle wound, care being taken to include all the layers. Pre-sterilized OOOO chromic cat-gut (Ethicon W548) was used for all buried sutures.

During pilot studies it was noted that many of the animals gnawed their skin sutures, particularly at the upper wound which often became patent. The situation was worse following the use of local anaesthetic solutions. The most satisfactory method found for suturing the upper wound was the use of double sutures. These raised a ridge of skin which protected the suture knot (Figs. 7, 8) and prevented the animal from gnawing the sutures.

The lower skin wound was repaired with a series of interrupted sutures tied securely (Figs. 7, 9). Occasionally the animals removed these sutures but if this happened they were promptly replaced.

Pre-sterilised white OOO silk (Ethicon W.509) was used for all skin sutures.

On completion of surgery the wounds were again swabbed with disinfectant solution and each animal was marked with an identifying number.

Urinary /

Urinary incontinence was common for two to three days after operation, but thereafter the animals recovered normal bladder control. Loss of weight was also observed following surgery, but the operation weight was regained within about six days and normal weight gain followed. The skin sutures were removed seven days after operation and about three weeks after operation the skin contour had returned to normal.

An attempt was made to standardise the transplant by attention to several variables:

Bladder size - Since, at the time of operation, all the animals were females of a similar age and weight, it was assumed that the bladders were of similar capacity. The bladder was often found to be distended and in an attempt to produce comparability of transplant size some of the urine was first expelled by gentle pressure before ligaturing.

Ligature position - The only satisfactory method found for defining the ligature position was that described by Huggins (1969), the ligature being always positioned just distal to the junction of the two large nerves on the ventral bladder surface.

Transplant size - The portion of bladder removed was approximately 5mm in diameter but, after removal, this tissue contracted slightly. Inevitably, while suturing the transplant to the anterior abdominal wall musculature, some tension was produced within it. The transplant /

transplant size, usually about 6 x 6mm when sutured in position, was always a compromise between the desirability of a standard size and shape, and the tension produced within the tissue.

HISTOLOGICAL PROCEDURES - Tissue removal

Light Microscopy

The animal was first anaesthetised using the same technique employed during transplantation, and the skin over the transplant shaved. The skin was incised on three sides of a square, and separated from the transplant/cyst roof by sharp dissection (Fig. 10). The transplant/cyst and surrounding muscle was then exposed, allowing the transplant/cyst with superficial muscle layers to be dissected free, and placed in fixative at $4^{o}C$. The animal was then killed by decapitation.

Specimens for histology were killed at -

Day 0 (I.P.O.*)	(3)	Day 17	(3)
Day 2	(3)	Day 20	(3)
Day 4	(3)	Day 25	(3)
Day 6	(3)	Day 30	(3)
Day 8	(3)	Day 40	(3)
Day 10	(3)	Day 100	(3)
Day 12	(3)	Day 180	(3)
Day 14	(3)		

Electron Microscopy - Scanning and Transmission

The animal was first anaesthetised, shaved and cyst exposed as described above. Two 25 gauge x 3/8" hypodermic needles attached to fine plastic tubing, were then /

* I.P.O. = Immediate Post Operative

then inserted through the cyst roof into the cyst lumen. Particular care was taken to avoid any contact with the cyst floor or walls. One tube was open at the far end, but elevated about 40 cms above the cyst, to maintain intracystic pressure. The other tube was directly connected to a 2ml hypodermic syringe, which was filled with fixative solution. The cyst lumen was next flushed very gently with fixative avoiding any distension of the cyst. After 10, and 20 minutes, the fixative within the cyst was replaced by further flushing. After 30 minutes the needles were removed and the animal killed by decapitation. The cyst roof was carefully cut off in the plane of the needle punctures and placed in fixative at 4°C, then the rest of the cyst and superficial layer of muscle were also removed in one piece and placed in fixative at 4°C.

The Day 9-11 specimens were fixed by perfusion of the whole animal through the left ventricle, because of the difficulty in locating the cyst lumen at this stage.

Specimens for transmission electron microscopy at -

Day 9	(1)	Day 16	(2)
Day 10	(5)	Day 20	(1)
Day 11	(2)	Day 40	(1)

Specimens for scanning electron microscopy at -

Day 6	(1)
Day 13	(1)
Day 30	(3)

All animals were killed at the same time of day as the operation, usually between 10.00 hrs. and 16.00 hrs.

Tissue Processing

Light Microscopy

1. Fixation - 10% phosphate buffered formalin for 48 hours at 4°C, pH 7.2
2. Decalcification - Equal parts, 50% Formic Acid and 20% Sodium Citrate for 48 hours* at room temperature. (only blocks 12 days post-operative or more).
3. Dehydration and Embedding - Standard method, through alcohols, benzene and paraffin wax.
4. Sectioning and Mounting - Each block was sectioned serially at 90° to the plane of the anterior abdominal musculature at a thickness of 10µm. All sections were mounted on glass slides and every 50th section indicated.

Electron Microscopy - Transmission

1. Fixation - 2.5% Glutaraldehyde solution in 0.2 molar cacodylate buffer pH 7.2
   (a) 30 minutes in situ
   (b) 2-12 hours at 4°C
2. Wash - 7.5% Sucrose in 0.2 molar cacodylate buffer pH 7.2. Two changes.
3. Post Fixation - 1% Osmium tetroxide in 0.2 molar cacodylate buffer pH 7.2. Two hours at room temperature.
4. /

* Day 100 and Day 180 specimens for six days.

4. Dehydration and Embedding -

10% Alcohol: 60 mins. - 2 changes

100% Alcohol: 90 mins. - 3 changes

Epoxy Propane: 30 mins. - 1 change

Epoxy Propane/Araldite: overnight

Araldite: 24 hours - 20°C

Araldite: 48 hours - 60°C

5. Sectioning and Mounting - The entire cyst base embedded in araldite was cut into slices at 90° to the plane of the anterior abdominal musculature, by a water-cooled rotary diamond saw. Thick 1 micron sections were cut from the corresponding face of each slice allowing selection of areas for thin sectioning. Thin sections (silver-gold colour) were cut using glass or diamond knives on a Reichert OMU3 Ultramicrotome and mounted on uncoated copper grids.

Electron Microscopy - Scanning

1. As for Transmission
2. do.
3. do.
4. Dehydration - 10% Alcohol: 60 mins. - 2 changes
   40% Alcohol: 60 mins. - 1 change
   80% Alcohol: 60 mins. - 1 change
   100% Alcohol: 90 mins. - 3 changes
   Amyl Acetate: 30 mins. - 1 change
   Amyl Acetate: overnight
5. /

5. Critical Point Drying - The tissue was washed in liquid carbon dioxide, until no amyl acetate could be smelled in the vented gas, and then left to soak under pressure for 3-6 hours depending on the specimen volume. At the end of this period the tissue was washed again in liquid carbon dioxide and critical point dried. A Polaron E3000 Critical Point Dryer was used. (Courtesy of the Department of Restorative Dentistry, Edinburgh University).

6. Mounting and Coating - All tissue was mounted on aluminium stubs using silver colloidal glue and after storage for 24 hours over phosphorous pentoxide was coated with gold in a Polaron E5000 sputter coater. (Courtesy of the Department of Electrical Engineering, Edinburgh University).

Stains

Light Microscopy

The slides containing every 50th section were stained Haematoxylin and Eosin, the subsequent slide stained Periodic Acid-Schiff (P.A.S.) and the slides containing every 100th section were stained Van Gieson. Selected slides were stained for fibrin using the M.S.B. (Martius, scarlet, blue) technique of Lendrum *et al* (1962), or the Osteoid stain of Ralis & Ralis (1975).

P.A.S. Technique

1. Sections brought to water
2. One half of slide immersed in 1% α-amylase in phosphate buffer pH7 for one hour at 37°C.
3. Rinse in water for five minutes
4. Other half of slide immersed in phosphate buffer alone for one hour at 37°C
5. Rinsed in water for five minutes
6. Oxidized in 2% sodium periodate for 10 minutes at 20°C
7. Rinsed in water for five minutes
8. Stained with Schiff's reagent (De Tomasi) for 30 minutes at 20°C
9. Rinsed in water for five minutes
10. Counterstained Delafields Haematoxylin 30 seconds
11. Dehydrated and mounted

Control Tissue - Freeze dried, paraffin embedded sections of rat liver

Positive Controls - Steps 1 and 6-11

Negative Controls - Steps 1, 2, 3, and 6-11

Electron Microscopy

All sections were stained on copper grids

1. 0.2% Lead Citrate in distilled water for two minutes
2. Rinsed in distilled water
3. Saturated solution of Uranyl Acetate in 50% Alcohol for 10-15 minutes
4. Rinsed in 50% Alcohol
5. Air dried

The preparations were viewed in a Phillips EM301 for transmission electron microscopy, or a Cambridge Stereoscan Mk II for scanning electron microscopy. (Courtesy of the Department of Electrical Engineering, University of Edinburgh).

MORPHOMETRIC ANALYSIS

Cyst Floor Mapping

Maps of the cyst floor were produced from tracings of every 50th serial section (vide Appendix A). Before tracing, each slide was examined under the microscope to prevent errors due to incorrect identification. The slides were projected on to a bench from a Leitz Prado Microprojecter via an eyepiece prism; the resultant magnification was about 30x. The profile of the cyst floor from edge to edge was traced on a sheet of white paper and the position of the features under investigation were marked.

Each tracing was measured by the method illustrated in (Fig. 11). First a base line (A-B) was drawn parallel to the plane of the anterior abdominal musculature, then a set of lines at 90^{o} to the base line were drawn to intersect each limit of the structures marked on the tracing (lines A, B, C. etc.). The distance along the base line to each intersection from the end of the base line, was then measured using a millimeter ruler (i.e. A - B, A - C etc.). The measurements from each section tracing were plotted on to a strip of graph paper at the same scale and the strips aligned side by side, to produce a map. Features common to adjacent sections were used as a guide to correct alignment of the strips. This alignment was not critical since all measurements were made on the individual sections and not on the completed /

completed maps. Any inaccuracy in alignment would only affect visual impressions from the completed maps. (These maps were a planar projection of a curved surface and therefore some compression of features occurred towards the periphery). The measurements from each tracing were also used to calculate the areas of the cyst floor maps, which were underlain by bone and/or smooth muscle. The results obtained were then analysed for statistically significant trends. The extent of the smooth muscle in the cyst floor could only be reliably discerned in Van Gieson (V.G.) stained preparations, and so the smooth muscle area, and results dependent upon this value were calculated using the data from only the V.G. stained sections.

A problem associated with the above technique was the lack of reliability with which the cyst edge could be identified, especially from Day 12 onwards. Before Day 10 the edge of the transplant was usually obvious and corresponded to the junction of the cyst floor and walls. However after Day 12 the following criteria had to be used: the floor edge was defined as that point on the cyst lining at which a tangent to the cyst lining made an angle of 45^{o} with the abdominal musculature plane.

Calibration

The projection system was calibrated prior to each set of drawings, by projecting a Zeiss calibrated slide, bearing a 2mm circle divided into quadrants by two crossed lines. The two diameters formed by the crossed lines were /

were measured and if their lengths were equal, the proportions of the projected image were considered to be correct. Magnification was calculated by measurement of the projected circle.

Section measurement

A Kontron, Manual Optical Image Analysing System was used. This system had two parts; the first was a display unit with 24 channel memory coupled to a printer output, the second part consisted of a measuring tablet and cursor, plus an electronic interface unit. Measurements were made on the tablet, using the cursor to trace the outline of the object being measured. The system sensed the position of the cursor on the tablet and computed the area outlined, and the length of the line traced. This data was automatically stored and displayed as a cumulative total, at the end of each measurement. The resolution of the tablet was 0.2mm.

The measurements were made on every 50th section, i.e. at 500 µm intervals through each block (vide Appendix A). These sections were viewed during measurement either by the same method as that described for cyst floor mapping, using a Leitz micro-projector, calibrated in the same way (Fig. 12) or using a Leitz SM Lux microscope fitted with a drawing tube, which allowed simultaneous viewing of the slide and tablet with its cursor (Fig. 13).

This second method was calibrated by viewing simultaneously the Zeiss calibration slide under the microscope, and a millimeter ruler on the tablet, and

was /

was only used for the specimens from Day 100 onwards and I.P.O. specimens. The first method was used for all specimens between Day 2 and Day 40.

Measurements were made of the following:

Cyst lumen

Bone beneath the cyst floor epithelium

Spaces within the above bone

Epithelial outgrowths in cyst floor and roof

Bone associated with these outgrowths

Bone beneath the cyst roof epithelium

The data from the measurement of each of the sections from one cyst was totalled for each feature, and corrected for magnification. The corrected totals (vide Appendix B) were then analysed for significant trends. Only the data for bone spaces could be reliably extrapolated to three dimensions using stereological principles (vide Appendix C). Mean sectional bone thickness (M.T.B.) was calculated from the bone sectional area total (F.B.S.A.T.) and the area of cyst floor underlain by bone as measured on the maps (B.F.A.).

$$\text{i.e. M.T.B.} = \frac{\text{F.B.S.A.T.} \times \text{Sampling interval}}{\text{B.F.A.}}$$

Method of measurement

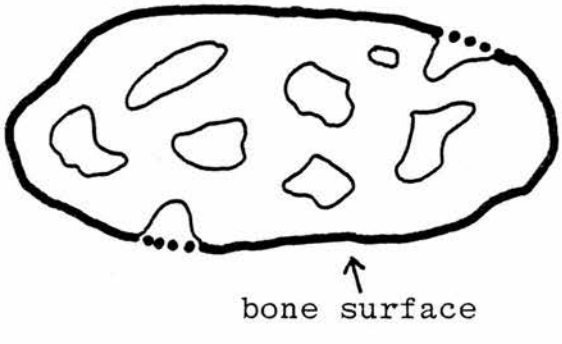
Cyst lumen

The entire luminal surface of the cyst lining epithelium of all cysts was traced in every fiftieth section and the linear measurements so obtained were summuted (C.P.T.). In isolated areas of severe epithelial hyperplasia a line was projected through the hyperplastic epithelium at the level of the luminal surface of the surrounding non-hyperplastic epithelium, and this line formed part of the measurement.

Bone beneath cyst floor or cyst roof epithelium or related to outgrowth epithelium.

The periphery of the bone was traced (heavy line) except in areas where foraminae were present. In such areas a line was projected between adjacent parts of the bone surface (dotted line).

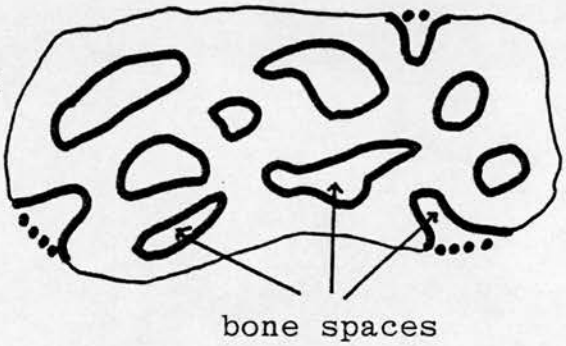
Diagram of a section through bone in the cyst floor



Spaces in bone beneath the cyst floor.

The margin of all internal bone spaces except individual lacunae, was traced (heavy line). Where spaces opened onto the external bone surface, the tracing was limited to the heavy line in the diagram, and for area measurements a line was projected (dotted line) in line with the external bone surface.

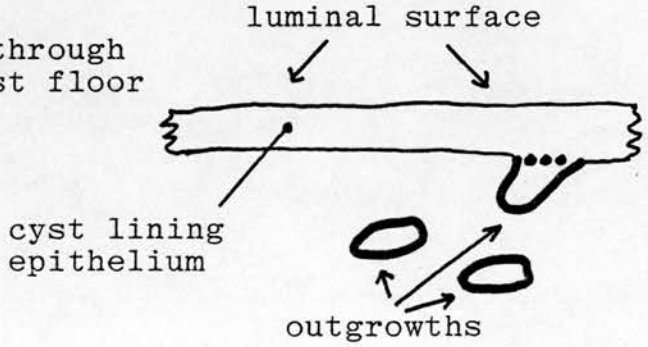
Diagram of a section through bone in the cyst floor



Epithelial outgrowths in cyst floor and roof.

The interface between outgrowth epithelium and the surrounding connective tissue was traced (heavy line). When an outgrowth was continuous with the cyst lining epithelium, to enable area measurement a line was projected (dotted line) through the junction of outgrowth and cyst lining epithelia.

Diagram of a section through the tissues of the cyst floor



CHAPTER 3

CYST FORMATION - DAY 0 - DAY 10

RESULTS - TRANSPLANT

MACROSCOPIC APPEARANCE

At operation the peritoneal surface of the transplant was pink, with a smooth glistening surface, through which the larger blood vessels were visible. The mucosal surface was redder in colour, with a velvety texture. When sutured in place the transplant was flattened peripherally, but centrally it was often raised to form a dome shape. The transplant had taken on a grey-blue colour by Day 2; its edge was still clearly visible, and could be easily separated from the site tissues. A haemorrhagic exudate was present around the transplant. By Day 4 some swelling was seen within the transplant and its edge was much more difficult to identify. At Day 6 the haemorrhagic exudate began to dissipate, and as the blood supply was restored, the swelling within the transplant declined up to Day 10.

MICROSCOPIC APPEARANCE

Day 0 = Immediate post-operative

The transplant was very similar to non-distended bladder wall '*in situ*'. The epithelium was typically three cells deep with a gradation of cell appearance from the superficial cells which were the largest and often binucleate, to the basal cells which were smaller with much less cytoplasm. Directly beneath the epithelium lay a /

a dense fibrous lamina propria, which was very cellular and contained many capillaries close to the basal layer of the epithelium. Beneath the lamina propria merging with it, lay a less dense submucosa, which contained larger blood vessels and a few nerves (Figs. 14, 15). The muscle coat formed most of the thickness of the transplant and consisted of smooth muscle fibres which apparently did not lie in any distinct planes or layers. A thin fibrous capsule was seen covering the peritoneal surface of the transplant.

When stained with P.A.S. glycogen was readily seen, mainly within the superficial cells of the epithelium, but the concentration varied considerably between different areas of epithelium. If the sections were first incubated with amylase a small amount of amylase resistant material could be detected in the luminal part of the superficial cells.

No glycogen was observed in the smooth muscle coat.

Day 2 - Day 10

Epithelium

At Day 2 the epithelium was reduced in thickness; and by Day 4, in some central areas, was only one squamous cell deep (Fig. 16). After Day 6, in these areas, the epithelium began to increase in thickness, and was about two cuboidal cells deep by Day 10 (Fig. 17). Peripherally the epithelium rapidly regained a normal appearance and thickness and after Day 6 some parts became hyperplastic (Fig. 18). /

(Fig. 18). When stained with P.A.S. at Day 6, large glycogen granules were found in almost all the surface cells. These granules became reduced in size and concentration thereafter, and by Day 10 were small and sparsely distributed. A few small outgrowths of epithelium into the lamina propria and submucosa of the transplant, were found from Day 6 onwards. These proliferations were most often found near the periphery of the transplant (Fig. 19).

Lamina Propria

Cellular necrosis was observed in the lamina propria from Day 2 (Fig. 20) and by Day 4 essentially the only cells which could be found within this layer were white blood cells, except close to the periphery of the transplant. In the areas of severe necrosis towards the centre of the transplant, even the collagen was removed (Fig. 21). By Day 8 in those areas in which the collagen had not been removed, the lamina propria was converted into a zone which was acellular (except for a few white blood cells) and filled with a homogeneous material, which gave the lamina propria a hyaline appearance (Fig. 22). Although the collagen fibres were obscured by this material in the Haematoxylin and Eosin stained preparations, when Van Gieson's stain was used, these fibres were readily seen.

Submucosa

From Day 2 an increasing amount of oedema was seen within /

within the submucosa associated with blood-filled spaces. The oedema was most marked at the centre of the transplant, where the submucosal tissue architecture was completely disrupted, so that the epithelium and lamina propria were separated, by the swelling, from the smooth muscle coat (Fig. 21). Due to this swelling the surface of the transplant lost most of its folds, and became smooth, with only a few narrow clefts, which divided up the surface of the transplant. At Day 6 the swelling in the submucosa was maximal, and slowly regressed thereafter.

Muscle coat

Necrotic changes were also seen in the inner layers of smooth muscle from Day 2, and by Day 6 had spread to involve most of the smooth muscle coat. Apparently normal muscle was only present peripherally, and in a narrow zone, against the peritoneal surface of the transplant (Fig. 17). When stained with P.A.S. the more normal areas contained many fine granules of glycogen on Day 2 and Day 4 (Fig. 23). The whole transplant was infiltrated with white blood cells from Day 2, initially, mainly lymphocytes, but by Day 6 polymorphonuclear leucocytes and plasma cells were also present. Thereafter the cellular infiltration subsided, as granulation tissue spread in from the periphery of the transplant.

SCANNING ELECTRON MICROSCOPIC APPEARANCE

At Day 6 when the epithelial surface of the transplant was viewed macroscopically, the swelling within the submucosa /

submucosa was found to have produced three or four hemispherical elevations of the mucosa, with narrow clefts between them. This arrangement was clearly seen under the Scanning Electron Microscope (Fig. 24).

When the epithelial cells which covered these elevations were examined at a greater magnification, several differences from bladder epithelial cells '<u>in situ</u>' were noted. The luminal plasma membrane of bladder epithelium '<u>in situ</u>', had a crumpled appearance (Fig. 25), and a few short, stubby microvilli could be seen on the cell surface. In contrast the cells of the Day 6 transplant, had an almost smooth surface, with a greatly increased number of the microvilli on the cell membrane, giving it a velvety appearance (Fig. 26).

RESULTS - TRANSPLANT SITE

MICROSCOPIC APPEARANCE

Day 0

Immediately following operation, the site tissues were very little changed. A very narrow layer of fibrous tissue covered the surface of the striated muscle and a few scattered epithelial cells were found on the surface of this layer (Fig. 27). Between the fibrous layer and the muscle a few small areas of adipose tissue were found. At the edge of the site (in about 5% of the sections examined), a flap of transplant mucosa was seen. This flap overlapped on to the transplant/site and contained no smooth muscle (Fig. 28).

Day 2 - Day 10

Epithelium

The new growth of epithelium from the edges of the transplant, across the site tissues, was first seen at Day 2 (Fig. 29). Thereafter the epithelium spread rapidly; the better the blood supply to the transplant edge, the more extensive was the epithelial proliferation across the site surface (Fig. 30). The new epithelial growth had completely covered the site by Day 10.

The epithelium was usually 2-3 cells deep, but varied especially at the growing edge; from about three cells deep with a rounded growing edge (Fig. 31), to a tapered sheet only one cell deep at its extreme edge (Fig. 32). Small islands of epithelial cells were also found on, and within /

within the superficial layers of the site from Day 2 (Fig. 33). These islands were a considerable distance from the growing epithelial edge, and were not connected to the epithelium proliferating from the transplant edge. The epithelium of the completed cyst floor varied so much that no definitive description could be given. In some areas the epithelium was a layer 2-3 cells deep in which no cell specialization could be seen (Fig. 34); in other areas a multilayered epithelium was found which contained a gradation from small basal cells to large superficial cells as well as a hyperplastic epithelium with large surface cells which were bulbous in form (Fig. 35) and sometimes contained intra-cellular vesicles.

When stained with P.A.S. deposits of glycogen were seen in the proliferating epithelial cells from Day 6 (Fig. 36). These were similar in distribution and size to those seen in the transplant epithelium at this time. By Day 10 these glycogen granules had almost disappeared.

Small pointed epithelial outgrowths from the basal layer into the underlying tissues, were observed from Day 4 (Fig. 37). These outgrowths apparently increased in number up to Day 10; they were usually small and did not normally extend deeply into the subepithelial tissues.

Other site tissues

On Day 2 a thin fibrin clot had formed, apparently from the site tissues, which separated the epithelium of the transplant from the fibrous layer on the site surface (Fig. 38). The fibrin clot was characteristically stained /

stained with the M.S.B. stain for fibrin (Fig. 39), and became reduced in thickness towards its periphery. The underlying fibrous layer was increased in thickness but did not appear to contain any more fibrous tissue than immediately post-operative but had an expanded appearance. By Day 4 a large number of white blood cells, mainly polymorphonuclear leucocytes, were seen throughout the clot and fibrous layer (Fig. 16). The fibrin clot and fibrous layer no longer met at a distinct boundary but the two layers appeared to merge with each other. Granulation tissue was advancing into the clot by Day 6, with many macrophages and fibroblasts. When stained with M.S.B. the upper layers of the clot still stained characteristically for fibrin, particularly in those areas of site tissue not yet covered by epithelium. The tissues beneath the epithelial new growth had become more dense and fibrous in appearance. By Day 10 very little fibrin could be detected and most of the site tissues had the appearance of fibrous connective tissue. However, when stained with Van Gieson stain only the tissue close to the striated muscle stained in a manner characteristic of collagen. The fibrillar tissue beneath the epithelium was essentially orientated parallel to the epithelial surface, running in various directions in that plane.

Up to Day 10 no changes were observed in the striated muscle.

SCANNING /

SCANNING ELECTRON MICROSCOPY

Epithelium

At Day 6, when examined at low magnification, the epithelial spread across the transplant site was found to have formed three or four semi-circular growth fronts. These areas of new epithelium corresponded closely in position to the position of the swellings within the transplant submucosa. The epithelium was apparently growing preferentially in areas where the swellings were in contact with the site tissues. When the epithelial cells were examined at a higher magnification, small bumps could be seen on the cell membranes which appeared to be related to inclusion bodies (Fig. 40) similar in size to the glycogen granules seen in the light-microscope preparations. At greater magnification the cell surface was similar in appearance to that of the transplanted epithelium showing a smooth velvety surface covered with many short microvilli (Fig. 41).

Other site tissues

When the non-epithelial covered areas of the Day 6 site were examined, the fibrillar nature of the fibrin clot could be clearly seen, with the associated white cell infiltration (Fig. 42).

CYST FORMATION

The immediate post-operative condition showed the transplant lying against the site tissues peripherally, and a space between them centrally (Fig. 43). By Day 4, the attachment between transplant and site at the edges was sufficiently strong to prevent separation during processing for histology. Evidence of some fluid could be seen between transplant and site in histological preparations from this stage onwards (Fig. 44). The swelling within the transplant submucosa reached a maximum at Day 6. The forming cyst became palpable through the skin at about this time. Thereafter the accumulation of fluid within the forming cyst appeared to match the reduction in the swelling within the transplant so that by Day 10 the forming cyst, on palpation, felt a similar size to that at Day 6, but had considerably increased in volume (Fig. 45).

A sheet of epithelium was found, trapped between the transplant edge and site surface from Day 2 (Fig. 46). This sheet had begun to break up into strands by Day 6 and by Day 10 some of these strands had spread away from the interface between transplant and site into the surrounding tissues (Fig. 47).

RESULTS - MEASUREMENTS

SECTION MEASUREMENTS

(Vide Table A for measurements
Table C for graph data)

'Cyst' lumen perimeter length total (C.P.T.)

The C.P.T. (Fig. 48) of the forming cyst decreased to about a third of the I.P.O. value by Day 10 on the regression line. C.P.T. (or the length of luminal epithelial surface per section totalled) was used as a reference standard for other measurements and so account had to be taken of any change in C.P.T. when these measurements were analysed.

Epithelial outgrowth sectional area total (Outgrowth S.A.T.)

There was little outgrowth of epithelium before Day 4 (Fig. 49). Thereafter the Outgrowth S.A.T/C.P.T. ratio increased and by Day 10 was three times the value at Day 4. This was after account had been taken of changes in C.P.T. and the areas of site tissues not covered by epithelium.

'Cyst' floor area covered by epithelium (E.F.A.)

The proportion of the cyst floor covered by epithelium rapidly increased after Day 2 and the whole cyst floor was covered by epithelium shortly after Day 8 (Day 8.35 on the regression line). (Fig. 50).

CHAPTER 4

CYST DEVELOPMENT AND BONE FORMATION - DAY 10-DAY 25

RESULTS - CYST ROOF

MACROSCOPIC APPEARANCE

At Day 10 the cyst roof was r e d and swollen, but by Day 25 the inflammation had gone and the roof was pink, with a central translucent area. At Day 10 there was vascular granulation tissue around the cyst, but by Day 25 this had become less vascular and more fibrous. This fibrous tissue was adherent to the cyst roof at its periphery, but more readily separated from it centrally.

MICROSCOPIC APPEARANCE

Epithelium

The epithelium lining the cyst roof was still thin centrally at Day 10. It was usually about two cells deep but towards the periphery of the roof it was much closer in appearance to '*in situ*' epithelium, and occasionally mildly hyperplastic. Generally the epithelial thickness seemed to reflect the state of the underlying tissues, since a progressive increase in epithelial thickness was observed in concert with loss of oedema and restoration of the blood supply within the sub-epithelial tissues. However an occasional raised area of epithelial hyperplasia with a connective tissue core was found similar in appearance to a papilloma of the bladder wall (Fig. 52). By Day 25 the roof was lined by an epithelium similar to '*in situ*' epithelium but the surface cells were smaller and no folds were seen /

seen (Fig. 53).

A few small outgrowths from the lining epithelium into the underlying tissues were seen between Day 10 and Day 25, usually not extending into the smooth muscle coat.

At Day 10, strands of epithelium were also found external to the muscle coat, close to the interface (Fig. 54). These spread between the muscle coat and subcutaneous tissue and by Day 25 could be found external to any part of the cyst roof (Fig. 55). At Day 25 the epithelium in these outgrowths, usually had formed small spherical islands of epithelial cells.

Non-epithelial tissues

Although the tissues beneath the epithelium were still oedematous at Day 10, especially centrally, by Day 14 this oedema had usually subsided. By Day 17 the sub-epithelial tissues had been replaced by a layer of fibrous tissue, which was considerably less vascular than the original tissues. Peripherally a layer of hyaline material could be seen immediately beneath the epithelium. Beneath the fibrous zone, the remains of the muscle coat could be seen. Centrally this layer had often been reduced to a quarter of its original width, but increased in thickness progressively towards the periphery (Fig. 56).

SCANNING ELECTRON MICROSCOPIC APPEARANCE

One specimen was examined at Day 13. At low magnifications the luminal surface of the cyst roof was smooth /

smooth with no folds. At higher magnifications, the cell surfaces appeared crumpled, and a regular pattern of cell junctions was seen (Fig. 57). The surfaces of the individual cells were covered by small short ridges and a few short microvilli, between which invaginations of the cell membrane were visible (Fig. 59).

These features resembled those seen on the surface of moderately distended bladder epithelium '_in situ_'. However, fewer microvilli and more marked ridges were noted on the '_in situ_' epithelium (Figs. 58, 60).

RESULTS - CYST FLOOR

MACROSCOPIC APPEARANCE

The cyst floor had a pink, smooth surface, up to Day 17, but thereafter, where the periphery of the underlying bone elevated the epithelium, a shallow ridge could be seen. Small radial ridges were also seen, from around Day 20 onwards, which extended from the margin of the floor up the wall, and partially subdivided the cyst cavity.

MICROSCOPIC APPEARANCE

Epithelium

At Day 10 the epithelium which covered the cyst floor had a smooth surface and was usually about three cells deep (Fig. 61), except in areas of hyperplasia; by Day 25 its thickness had reduced slightly, especially in areas of the floor not related to bone formation.

Two forms of hyperplasia were found in the floor epithelium:

Hyperplasia A - a mild form which featured bulbous surface cells and moderate thickening of the epithelium (Fig. 62).

Hyperplasia B - a papillomatous form showing tufts of epithelium from which many surface cells were being exfoliated (vide p. 119). Unlike a true papilloma Hyperplasia B did not contain a connective tissue core (Fig. 63).

Hyperplasia A was widespread at Day 10 but by Day 25 had reduced to a few small central areas. The more severe /

severe Hyperplasia B was not seen until Day 14. Thereafter a few localized areas were commonly present near the centre of the cyst floor, often surrounded by a narrow zone of mild hyperplasia.

As with the cyst roof, outgrowths of epithelium were found within the interface and floor tissues between Day 10 and Day 25; only rarely were they continuous with the cyst floor epithelium after Day 14. At Day 10 the epithelial strands were congregated close to the margin of the floor. By Day 25 the epithelium was in the form of discoidal islands, expanded parallel to the cyst lining. These islands had apparently moved nearer to the centre of the floor (Fig. 64) close to the edge of the smooth muscle sheet (see below).

Small diverticuli confluent with the main cyst were occasionally seen in the interface between Day 10 and Day 25.

Sub-epithelial tissues - non-osteogenic areas

At Day 10 small peripheral areas of the cyst floor were underlain by smooth muscle the extent of which could only be discerned clearly in Van Gieson stained preparations (Figs. 65, 66). These areas steadily increased in size up to Day 25. This smooth muscle was usually a thin layer, only a few cells deep, which increased in thickness closer to the periphery of the floor. Those areas of floor supported by smooth muscle usually showed hyaline material beneath the epithelium, but by Day 25 the /

the hyaline areas were less distinct. No changes were seen in the striated muscle between Day 10 and Day 25.

From Day 12 onwards shallow ridges with a core of connective tissue were found on the cyst lining. The ridges extended radially from the edge of the floor up the wall, becoming more prominent with time, so that by Day 20 they were visible macroscopically when the cyst was dissected.

TRANSMISSION ELECTRON MICROSCOPIC APPEARANCE

At Day 10 the epithelium of the cyst floor was of variable thickness, with several short projections of the basal layer into the connective tissues. The epithelial surface was not flat, each cell producing a small elevation. In many areas these elevations developed a bulbous polypoid form.

Regions of epithelium were also found deeper within the floor tissues, and some of these outgrowths were so small (only 2-3 cells in diameter) that they would be difficult to identify under the light microscope. A few outgrowths were seen adjacent to, or within, osteogenic areas.

The epithelium showed little differentiation into distinct cell types, all the cells being similar to those found in the intermediate layer of '*in situ*' bladder epithelium (Fig. 67). The outgrowth epithelium was similar but the cells were usually smaller than those in the surface epithelium.

The surface of the superficial cells of the cyst floor was studded with short microvilli, and many small round /

round vesicles could be seen within the cytoplasm directly beneath the cell membrane (Fig. 68). Both these features were rare in bladder epithelium ' in situ'. A clear basal lamina, showing well-defined laminae lucida and densa, was always present beneath the lining epithelium, but was much less distinct around outgrowths at Day 10. The epithelium of the cyst floor had become similar in appearance to typical bladder epithelium 'in situ' by Day 20. The typical angular contour of the luminal surface, a few stubby microvilli and underlying fusiform vacuoles could be seen (Fig. 69). Furthermore differentiation into distinct cell layers was found within both the surface and outgrowth epithelium.

SCANNING ELECTRON MICROSCOPY

At Day 13 two types of epithelium were found.

Type 1 was smooth and flat with an irregular pattern of cell junctions, and the occasional polypoid cell protruding from the surface (Fig. 70). This epithelium was usually found towards the periphery of the floor.

Type 2 was usually found centrally and corresponded to mild Hyperplasia A with a surface formed by bulbous polypoid cells, some of which were elongated in a radial direction from the floor centre (Fig. 72).

The smooth flat cells of Type 1 had a cell surface which possessed many short microvilli, interspersed by invaginations of the cell membrane (Fig. 71); whereas the polypoid cells of Type 1 and Type 2 in general possessed a cell surface similar to that of 'in situ' epithelium, /

epithelium, showing few micro-villi and invaginations and exhibiting an overall crumpled appearance (Figs. 71, 73, 74).

RESULTS - BONE FORMATION

MICROSCOPIC APPEARANCE

Bone formation related to cyst floor epithelium

The first signs of bone formation were found at Day 10, very close to, but not in direct contact with the floor epithelium. The osteogenic zones were first visible as deeply basophilic areas associated with basophilic cells containing round polar nuclei. The fibres in these areas were readily stained by the Van Gieson method, in contrast to those in surrounding non-osteogenic zones (Figs. 75, 76). When stained with P.A.S. very small amylase-resistant granules, about 1 μm in diameter could be seen within the forming bone (Fig. 77).

When similar areas were examined at Day 12 the bone formation had spread parallel to the epithelial surface. In undecalcified sections the small P.A.S-positive amylase-resistant granules had enlarged and coalesced to form seams of P.A.S-positive amylase-resistant bone which was V.G-positive, and basophilic at the periphery. In decalcified sections the P.A.S. stained granules were absent, although the bone became V.G-positive throughout, and uniformly basophilic except in areas of osteoid.

After Day 12 undecalcified sections of the bone were still P.A.S.-positive amylase-resistant and the fine P.A.S-positive amylase-resistant granules first seen at Day 10 were still visible at Day 17 on growing bone surfaces.

In /

In decalcified sections the bone continued to be densely stained by the Van Gieson method after Day 12 but became less basophilic. The results with P.A.S. stain after decalcification were inconsistent and so considered unreliable (vide p.109).

The areas of bone formation spread rapidly thereafter to form sheets of cellular bone with many spaces. Plump osteoblasts could be seen on the bone surfaces and osteocytes in lacunae within the bone (Figs. 78, 79)

The collagen fibre pattern revealed by polarized light from Day 12 onwards showed that both before and after bone formation the majority of the fibres lay parallel to the epithelial surface (Figs. 80, 81).

No cartilage was found associated with the forming bone but occasional cellular areas, with a hyaline homogeneous matrix, were seen at the periphery of the bony sheet (Fig. 82). These areas were not cartilaginous: the cells were typical basophilic plump osteoblasts with polar nuclei, the matrix stained in a manner typical of osteoid when using the osteoid stain of Ralis & Ralis (1975) and no evidence of calcified cartilage could be seen at the interface between these areas and the bone itself. This material was osteoid.

The first sites of bone formation were found towards the edge of the floor in areas not related to smooth muscle. Bone formation spread preferentially (Day 12) towards the centre of that part of the cyst floor not underlain by smooth muscle. The bone then spread outwards /

outwards towards the edges of the cyst floor, and by Day 17 occupied most of the smooth muscle-free floor area. Between Day 17 and 25 little further lateral spread occurred (Fig. 83). The bony sheet so formed was initially thin especially at the edges but very rapidly increased in thickness (Fig. 84). This increase in thickness was accompanied by elevation of the floor epithelium, so that most of the bone formed was above the level of the surrounding floor. By Day 25 an elevated plaque of bone with rounded edges, and of fairly uniform thickness had formed (Fig. 85).

Spaces were seen within the new bone from Day 12. Initially the spaces were orientated at right angles to the epithelial surface (Fig. 78) but by Day 25 the bone spaces were larger and had become elongated parallel to the epithelial surface in many cases.

Typical multinucleate osteoclasts (Figs. 86, 87) were found mainly within the bone spaces from Day 17 onwards; reversal lines were also visible in the bone from this time. Osteoclasts were not evenly distributed over the bone surfaces but were found as localized groups. Large thin-walled blood vessels were also commonly seen within these spaces (Fig. 88).

A narrow zone of fibrous tissue was always present between the bone and the striated muscle. This zone became progressively reduced in thickness up to Day 25 (Fig. 88) as did the sub-epithelial tissues of the floor in non-bony areas.

Bone /

Bone formation related to outgrowth epithelium

From Day 10 bone formation of this type was found in the interface, and from Day 14 within the floor (Fig. 64) and external to the roof (Fig. 89). In all cases the histogenesis was similar to that described above. However, the volume of bone formed was generally much smaller than that formed directly beneath the floor epithelium. Osteogenesis usually occurred either as a ring of bone around an island of outgrowth epithelium (Fig. 90) or as a nodule of bone surrounded by a band of epithelium.

Areas of bone formation around outgrowths in the floor were rarely found after Day 20 due to such areas of bone having been incorporated into the extending floor bone (Fig. 91).

Bone formation related to cyst roof epithelium

From Day 17 bone could be found directly beneath the epithelium lining the cyst roof. This bone differed from that previously described. The quantity of bone formed was extremely small and it usually contained fewer spaces and cells (Fig. 92). The first bone formation of this type was found not directly beneath the roof epithelium, but beneath the hyaline zone which usually underlay the epithelium in these areas (Fig. 93). The bone formation only later spread into the hyaline zone and came to lie directly beneath the epithelium. From Day 17 onwards bone began to spread subepithelially in a few small areas, from the floor into the walls of the cyst. This /

This spread was often found at the base of the ridges on the cyst wall (previously described on p.63). These extensions of the floor bone came to underlie areas of hyaline material and the bone later extended into these areas (Fig. 94) in a similar manner to that described for bone formation in the cyst roof.

TRANSMISSION ELECTRON MICROSCOPIC APPEARANCE

From Day 9 onwards small groups of cells were found beneath the cyst floor epithelium. These cells contained a round or oval nucleus usually polar in position which featured one or two prominent nucleoli. Their cytoplasm contained an extensive granular endoplasmic reticulum, Golgi lamellar arrays, mitochondria, and some electron-dense vesicles (Fig. 95). These cells, presumably pre-osteoblasts, were separated by collagenous matrix in which cell processes could be seen.

The adjacent areas of non-osteogenic tissue contained fibroblasts separated by loose collagenous material. The fibroblasts were elongated cells containing less granular endoplasmic reticulum than the pre-osteoblasts (Fig. 96). By Day 10 the osteoblasts were further apart, probably due to the production of collagenous matrix (osteoid) by these cells (Fig. 97). Small 'coated' vesicles could be seen beneath the surface of some cells and other intracellular vacuoles which seemed to contain collagen (Figs. 98, 99). In the centre of the osteogenic zones, sites of initial calcification could be seen within the bands of osteoid (Fig. 100). This appearance was widespread by Day 11.

Three /

Three types of vesicular structures could be identified in the intercellular matrix from Day 10. Type 1 profiles were irregular in outline (0.1-0.25μm in diameter) with electron-lucent contents, and were assessed to be cell processes. Type 2 were usually round or oval, often smaller than Type 1 (0.05-0.2μm in diameter) with a variable mixture of electron-lucent and electron-dense contents. Type 3 were always round, usually larger than Types 1 and 2 (0.1-0.4μm in diameter) and had electron-dense contents. All types of vesicle were limited by a trilaminar membrane (Fig. 101). Type 3 vesicles were much less frequent than the other types.

The electron-dense material found in Type 2 vesicles was in the form of needle shaped crystallites (Fig. 102): these were assumed to be hydroxyapatite. Larger radial clusters of these crystallites were regularly found in the same areas as the vesicles. Occasionally some of the smaller clusters of apatite crystals could be seen to be centred on 'ghost' Type 2 vesicles (Fig. 103). It would appear that the initial apatite crystals formed within Type 2 vesicles. These crystals then enlarged and ruptured the vesicular membrane and thereafter crystal growth extended radially from the nucleus so formed.

On Day 11 similar appearances could be found at the edge of the osteogenic areas but towards the centre of these areas, the apatite clusters had enlarged sufficiently to coalesce and form seams of calcified bone.

The /

The apatite clusters reached approximately 1µm diameter before they coalesced.

At the edge of the osteogenic areas by Day 16, appearances similar to those described previously could be seen (Figs. 104, 105), with many apatite clusters being incorporated into the bone surface.

The collagen close to the osteoblasts was finer in texture and showed an increase in diameter as it approached the calcification front (Fig. 104). Closer to the centre of the osteogenic areas the spaces between the osteoblasts were usually calcified, with a narrow zone of uncalcified matrix close to the cell surface. Some of the osteoblasts were immured within the bone to become osteocytes. Narrow cell processes extended into the bone from these osteocytes (Fig. 106) which were often seen to contain aggregates of glycogen granules (Fig. 107) and also dark vesicles possibly secondary lysosomes. These dark vesicles (Fig. 106) were similar to those seen at Day 9 in the pre-osteoblasts, and within the intercellular spaces as Type 3 vesicles.

The osteocytes were usually separated from the surrounding bone by a narrow zone of uncalcified matrix.

RESULTS - MEASUREMENTS

SECTION MEASUREMENTS

(Vide Table A for measurements
Table C for graphs data)

Cyst lumen perimeter length total (C.P.T.)

Between Day 10 and Day 25 there was no statistically significant change in the C.P.T.

Bone sectional area total (Floor Bone S.A.T)

There was a marked increase in the S.A.T. of bone which formed in relation to the cyst floor epithelium from Day 10 (Fig. 108). On the regression line this value increased by $3.460 \times 10^{-3} mm^2/mm$ C.P.T. every 24 hours, up to Day 17. Between Day 17 and Day 25 no statistically significant change in Floor Bone S.A.T. occurred.

Bone spaces volume (Sp. Vol.)

The volume of bone spaces per unit bone volume, did not alter significantly between Day 12 and 25.

Bone spaces surface area (Sp. S.A.)

There was a significant reduction in bone spaces S.A. per unit bone spaces volume between Day 12 and 25 (Fig. 109). The reduction in the ratio was about 40% on the regression line.

Osteoclasts

No osteoclasts were found prior to Day 17. After Day 17 osteoclasts were seen mainly in bone spaces and there was no significant change in the mean number of osteoclasts per unit surface area of bone spaces.

Epithelial outgrowth S.A.T. (Outgrowth S.A.T.)

In /

In the case of epithelium which had grown away from the cyst lining the S.A.T. per unit C.P.T. diminished between Day 10 and 25 by 78% on the regression line (Fig. 110). When the data prior to Day 10 was included, it could be seen that the S.A.T. of outgrowth epithelium was maximal around Day 10 and by Day 25 had returned to a level close to that at Day 5.

Bone S.A.T. related to outgrowth epithelium
(Outgrowth Bone S.A.T)

The S.A.T. of bone formed adjacent to outgrowth epithelium increased relative to the S.A.T. of epithelium between Day 10 and 25 (Fig. 111). However, the increase in this ratio was partially due to the fall in Outgrowth S.A.T. between Day 10 and Day 25. In order to examine the real change in Outgrowth Bone S.A.T. during this period, the rate of change in Outgrowth Bone S.A.T. was calculated using a corrected Outgrowth S.A.T. (i.e. corrected to Day 10 when this is maximal). After this correction the Outgrowth Bone S.A.T. still showed an increase between Day 10 and Day 25 of $1.18 \times 10^{-2} mm^2/mm^2$ Outgrowth S.A.T. (corrected) every 24 hours. This is just over a quarter of the uncorrected rate of increase.

Bone S.A.T. related to cyst roof epithelium
(Roof Bone S.A.T)

Bone was found related to the roof epithelium from Day 17. The S.A.T. of bone formed did not show any significant change up to Day 25. Between Day 17 and 25 the average S.A.T. of bone per mm C.P.T. was $5.714 \times 10^{-4} mm^2$, compared with an average in the floor of $2.623 \times 10^{-2} mm^2$ for the same period, i.e. 46 times more beneath the /

the floor epithelium than the roof epithelium.

CYST FLOOR MAP MEASUREMENTS

(Vide Table B for measurements
Table C for graphs data)

Cyst floor area (C.F.A.)

There was no significant change in cyst floor area between Day 10 and Day 25.

Bone area (B.F.A.)

The proportion of the cyst floor underlain by bone formed next to floor epithelium, increased up to Day 17 (Fig. 112) by 5.14 x $10^{-2}mm^2$ per mm^2 cyst floor, every 24 hours on the regression line. Between Days 17 and 25 the proportion of the floor underlain by bone did not change significantly.

Bone thickness (M.T.B.)

The mean sectional thickness of the bone formed in relation to floor epithelium rose steadily up to Day 17 on the regression line (Fig. 113) and thereafter showed no significant change up to Day 25. The "floor" bone had a mean thickness of 95μm at Day 17.

Smooth muscle area (S.F.A*)

The proportion of the floor underlain by smooth muscle increased steadily from Day 8 on the regression line (Fig. 115). However, if this area underlain by smooth muscle was subtracted from the total floor area, the area of floor remaining showed no significant change up to Day 25.

Area not underlain by bone or smooth muscle (N.F.A*)

The /

* V.G. stained sections only

The proportion of the floor not underlain by smooth muscle or bone (Neither F.A.) rapidly decreased between Day 10 and 25, so that by Day 25, only 12.5% of the floor area was underlain by neither tissue,on the regression line (Fig. 116). Bone formation was usually restricted to the smooth muscle-free floor (Fig. 83) and when an overlap between bone and smooth muscle did occur, no more than 2% of the floor area was involved.

CHAPTER 5

FURTHER DEVELOPMENT IN CYST AND BONE - DAY 25-180

RESULTS - CYST ROOF

MACROSCOPIC APPEARANCE

The cyst roof between Day 25 and 180 progressively became thinner and more transparent, as the cyst enlarged. The cyst fluid, which could be seen through the roof, varied in colour from clear to dark-brown. Subdivisions of the cyst cavity peripherally, were visible in the intact cyst from about Day 40 onwards.

MICROSCOPIC APPEARANCE

Epithelium

The epithelium of the cyst roof at Day 25 was usually 2-3 cells deep, with an increase in cell size towards the luminal surface but considerable variation was found (Fig. 53). Between Day 25 and Day 180, as the cyst enlarged the epithelium slowly decreased in thickness. This reduction was most marked in those areas of the cyst roof which also showed considerable thinning of the smooth muscle (Fig. 117). The epithelium which overlay areas of broader smooth muscle, retained a more normal appearance (Fig. 118). By Day 180 almost the whole of the roof was lined by a squamous epithelium 1-2 cells in thickness (Fig. 119).

The number and size of epithelial outgrowths external to the cyst roof which had arisen from the interface between cyst wall and floor, appeared to reduce between Day 25 and Day 180, and by Day 180 were only infrequently seen. /

seen. Outgrowths from the cyst roof epithelium into the underlying tissues were very rarely seen after Day 25.

Sub-epithelial tissues

The main change in the rest of the cyst roof between Day 25 and Day 180 was the progressive thinning of the smooth muscle coat and the sub-epithelial fibrous layer, possibly due to the stretching of the cyst wall. The areas of hyaline material beneath the cyst roof epithelium became more and more indistinct, and by Day 180 were usually absent altogether.

Sutures

The sutures which were used to locate the transplant were resorbed very slowly, being still readily recognizable at Day 100. By Day 180 the sutures had been resorbed in most cases but occasional remnants could still be found (Fig. 117).

RESULTS - CYST FLOOR

MACROSCOPIC APPEARANCE

The ridges extending from the cyst floor up into the cyst wall, first seen at Day 20, gradually became more prominent between Day 25 and Day 180 as the cyst enlarged. These ridges had developed by Day 180 to such an extent that they subdivided the cyst cavity, into several confluent loculi. The margin of the bony plaque could be readily observed on the cyst floor as a raised edge. Areas of bone could be seen projecting above the main bone mass from Day 100.

The bone in the cyst floor could be detected radiographically in the intact animal from Day 30 onwards.

MICROSCOPIC APPEARANCE

Epithelium

At Day 25 most of the cyst floor was covered by a thin homogeneous epithelium about 2-3 cells deep. By Day 180 it was reduced a little to about two squamous cells deep. Virtually no mild Hyperplasia A was seen between Day 25 and 180, but papillomatous Hyperplasia B was usually present towards the centre of the cyst floor (Fig. 120). At Day 40 areas of the hyperplastic. epithelium appeared to be exfoliated (Fig. 121) leaving a portion of the cyst floor in which an epithelial layer was difficult to identify (Fig. 122, 123). Around the margin of these apparently denuded areas a narrow zone of Hyperplasia B remained (Fig. 122). This arrangement

was /

was still present at Day 180; Hyperplasia B often appeared to be infiltrated with many red blood corpuscles.

Outgrowths from the cyst lining were seen within the cyst floor from Day 25 onwards, but the size and number of these epithelial islands reduced gradually and by Day 180 very few were found. The outgrowths were usually congregated close to the edge of the smooth muscle layer beneath the cyst floor. A few of these epithelial outgrowths were seen beneath the edge of the bone in the floor, up to Day 30 (Fig. 124) but thereafter virtually none were seen in this position.

Sub-epithelial tissues

In non-bony areas the sub-epithelial tissues changed little in appearance between Day 25 and Day 180 apart from a gradual decrease in thickness (Figs. 125, 126). The epithelium was underlain by a layer of fibrous connective tissue which contained a sheet of smooth muscle, normal in appearance and of variable thickness. Directly beneath the fibrous layer the anterior abdominal musculature could be seen, although peripherally, areas of adipose tissue were interposed, from Day 100. No changes were observed in the striated muscle.

TRANSMISSION ELECTRON MICROSCOPIC APPEARANCE

At Day 40 the cells of the floor epithelium were generally similar in all areas to those of '*in situ*' epithelium. The epithelium was differentiated into the three cell types and the luminal cell membrane showed the typical fusiform vacuoles and angular membrane (Fig. 127).

A /

A distinct basal lamina was always found beneath the epithelium (Fig. 128). In areas of (papillomatous) Hyperplasia B, R.B.Cs were commonly found in the intercellular spaces at all levels within the epithelium (Fig. 127). A similar appearance could be found in non-hyperplastic areas but the R.B.Cs were much less common. At Day 40 many epithelial and sub-epithelial cells contained large vacuoles with homogeneous electron-dense contents similar to R.B.C. cytoplasm.

SCANNING ELECTRON MICROSCOPIC APPEARANCE

At Day 30, the surface of the lining epithelium was similar in all areas and had the typical crumpled appearance of '*in situ*' epithelium (Fig. 129).

RESULTS - BONE FORMATION

MICROSCOPIC APPEARANCE

Bone formation related to cyst floor epithelium

Bone was always present beneath the floor epithelium between Day 25 and Day 180. At Day 25 the bone was in the form of a plaque which contained many spaces and had elevated the floor epithelium (Fig. 130). In a few places, extensions of bone formation from the edge of the plaque up the cyst wall were seen.

The bone continued to grow slowly on both upper and lower surfaces up to Day 40, after which growth apparently ceased, except centrally directly beneath the floor epithelium. Here bone formation continued up to Day 180 resulting in a gradual increase in the thickness of the plaque in this area (Fig. 131). Prior to Day 30 the bone always closely underlay the floor epithelium, separated only by a single layer of osteoblasts but at Day 30, an area of fibrous tissue appeared centrally between the epithelium and the bone (Fig. 121.) By Day 40 this fibrous tissue had been converted to osteoid and the epithelium directly above it was exfoliated (vide p.81), (Figs. 122, 132). The zone of osteoid continued to enlarge slowly up to Day 180 when a mass of bone had been formed which projected well above the original bony plaque (Fig. 133). When stained with the osteoid stain of Ralis & Ralis (1975) the broad band of osteoid was deeply stained, and thin layers of osteoid were also stained /

stained on the surfaces of the bone spaces within the plaque (Fig. 134). The bone directly beneath the zone of osteoid appeared to mature rapidly to lamellar type bone (Figs. 133, 134).

Until Day 40 there was little evidence of osteone formation but at Day 100 a few primary osteones could be seen and by Day 180 the bone was typical lamellar bone, composed of a few circumferential lamellae surrounding some osteones (Fig. 135). When the bone was viewed in polarized light, much of the collagen was orientated parallel to the cyst floor but a few osteones showing a maltese cross pattern could be found from Day 100.

The bone spaces enlarged and coalesced, and by Day 40 produced a small central cavity (Fig. 136). At Day 180 this marrow space had enlarged into an extensive cavity, crossed by a few trabeculae (Fig. 133). Bone marrow cells were first found on Day 30 (Fig. 137) and by Day 40 were present in all bone spaces. Until Day 180 extensions of the floor bone were seen beneath the epithelium of the cyst wall. These extensions, like the bony plaque, elevated the epithelium to produce a shallow ridge on the cyst wall.

Bone formation related to outgrowth epithelium

Bone was found related to epithelial outgrowths in the roof (Fig. 138) and floor of the cyst between Day 25 and Day 180, resembling that found in similar situations between Day 14 and 25. The number and size of the areas of bone gradually reduced towards Day 180.

Bone /

Bone formation related to cyst roof epithelium

Bone also occurred as small isolated masses beneath the roof epithelium, between Day 25 and Day 180 (Fig. 139), again similar in appearance to that found between Day 17 and 25. These areas of bone also reduced in number and size towards Day 180.

TRANSMISSION ELECTRON MICROSCOPIC APPEARANCE

At Day 40 immediately beneath the epithelium, a single layer of osteoblasts could be seen, with associated capillaries. The osteoblasts were separated from the bone surface by a narrow layer of osteoid which contained cell processes (Type I vesicles) and apatite clusters. No Type 2 and 3 vesicles were seen. A gradation in collagen fibre diameter towards the calcification front was also observed (Fig. 140). Within the bone, osteocytes and their processes, often containing aggregates of glycogen particles, were surrounded by a narrow zone of osteoid (Fig. 141). Many haemopoetic cells and blood vessels were seen within the bone spaces, which were lined by a layer of osteoblasts which was occasionally deficient.

SCANNING ELECTRON MICROSCOPIC APPEARANCE

At Day 30 the areas of bone formation were readily identified as elevated portions of the cyst floor with a prominent edge (Fig. 142). The epithelium overlying the bone showed evidence of shrinkage, although non-bony areas were unaffected. Extensions of the bone formation /

formation from the floor up the cyst wall were clearly seen (Fig. 143) and centrally an area of Hyperplasia B was usually present.

RESULTS - MEASUREMENTS

SECTION MEASUREMENTS

(Vide Table A for measurements
Table C for graphs data)

Cyst lumen perimeter length total (C.P.T.)

Between Day 25 and 180 a significant rise in C.P.T. was found. Although no such trend had been detected between Day 10 and Day 25, when the data between Day 10 and Day 180 was analysed a significant rise in C.P.T. was again found (Fig. 144). The C.P.T. rose by 230% on the regression line between Day 10 and Day 180 (173%, 25-180).

Bone sectional area total (Floor Bone S.A.T.)

The S.A.T. of bone per unit C.P.T. beneath the cyst floor epithelium showed no significant change between Day 25 and Day 180 (Fig. 145). The S.A.Ts of Floor Bone, Outgrowth Bone and Roof Bone are compared in Fig. 146.

Bone spaces volume (Sp. Vol.)

The volume of spaces per unit bone volume in the cyst floor showed no significant change between Day 25 and Day 180. However, when the data between Day 12 and Day 25 was combined with the Day 25-Day 180 data, a significant increase was found (Fig. 147). The ratio rose by 38% on the regression line between Day 12 and Day 180.

Bone spaces surface area (Sp. S.A.)

The surface area of bone spaces per unit bone space volume decreased considerably between Day 25 and Day 180 (Fig. 148). The ratio fell by 56% on the regression line during this period.

Epithelial /

Epithelial outgrowth S.A.T. (Outgrowth S.A.T.)

The fall in Outgrowth S.A.T. already seen between Day 10 and Day 25 continued more slowly between Day 25 and Day 180 (Fig. 149). Outgrowth S.A.T. per unit C.P.T. fell by 97% during this period on the regression line. (After allowing for the increase in C.P.T. this still represented a fall of 95% in Outgrowth S.A.T. per unit C.P.T. (at Day 25).

Bone S.A.T. related to epithelial outgrowths (Outgrowth Bone S.A.T.)

There was no significant change in the S.A.T. of bone per unit outgrowth S.A.T. between Day 25 and Day 180.

Bone S.A.T. related to cyst roof epithelium (Roof Bone S.A.T.)

The S.A.T. of bone beneath the cyst roof epithelium did not change significantly between Day 25 and Day 180.

CYST FLOOR MAP MEASUREMENTS

(Vide Table B for measurements
Table C for graphs data)

Cyst floor area (C.F.A.)

Between Day 25 and 180 a significant rise in C.F.A. was found. Although no such trend had been detected between Day 10 and Day 25, when the data between Day 10 and Day 180 was analysed a significant rise in C.F.A. was again found (Fig. 150). The C.F.A. rose by 214% on the regression line between Day 10 and Day 180 (150%, 25-180).

Bone floor area (B.F.A.)

The /

The proportion of the cyst floor underlain by bone appeared to be maximal at Day 30, and had decreased 57% by Day 180 on the regression line (Fig. 151). However, during the same period (Day 30-Day 180) the cyst floor area increased by 157% so that the area of cyst floor underlain by bone per unit cyst floor area (at Day 30) increased by about 10%. This would suggest that after Day 30 further spread of bone formation was limited.

Bone thickness (M.T.B.)

The mean sectional thickness of the bone in the cyst floor rose by 316% between Day 25 and Day 180 to reach 390μm by Day 180 (Fig. 152).

Smooth muscle area (S.F.A.*)

The increase in the proportion of the cyst floor underlain by smooth muscle, seen between Day 10 and Day 25, continued between Day 25 and Day 180 (Fig. 153). (once again if this area was deducted from the total cyst floor area in each case, no significant change occurred in the remaining area between Day 25 and Day 180. No change was seen even if all the data between Day 10 and Day 180 were combined.) This proportion of the cyst floor, possibly derived from the cyst roof, rose by 142% on the regression line between Day 25 and Day 180, and at Day 180, 80% of the cyst floor was underlain by smooth muscle.

Area not underlain by bone or smooth muscle (N.F.A.*)

The proportion of the cyst floor not underlain by bone /

* only V.G. stained sections measured

bone or smooth muscle (Neither Floor Area) decreased between Day 25 and Day 180 (Fig. 154), continuing the trend seen between Day 10 and Day 25. By Day 180 on the regression line, only 1% of the cyst floor was not underlain by either tissue. (This represented only 0.2% of the smooth muscle-free floor). Overlap between areas of bone formation and areas of smooth muscle (O.F.A.*) was seen in less than half of the cysts examined between Day 25 and Day 180 and when found, averaged only 3% of the cyst floor area.

* only V.G. stained sections measured

CHAPTER 6

DISCUSSION - CYST

TRANSPLANT TISSUES

The early changes within the sub-epithelial tissues of the transplant, correlated well with those described in studies of the vascularization of free autogenous skin grafts. The only nutrition to the corium of the skin grafts during the first day was from a circulation of plasma within it (Goldman, 1894). This circulation was more effective if the blood vessels in the graft were not occluded (Davis & Traut, 1925). At the end of the first day, the re-establishment of a blood supply commenced, with the link-up of a few capillaries between graft and site. This early blood supply was not sufficient however to prevent necrosis within the graft corium. At Day 4 the new growth of capillaries into the graft began and by Day 8 had progressed sufficiently for repair processes to commence (Davis & Traut, 1925; Mir y Mir, 1951).

In the present investigation the restoration of the blood supply followed a similar pattern. The peripheral tissues of the transplant and those close to the serosal surface survived, presumably by plasma circulation. However necrosis occurred in the central part of the transplant accompanied by extensive oedema within the submucosa. Repair of areas of necrosis was observed from about Day 6 onwards probably associated with the restoration of blood supply. Later, when the cyst roof

was /

was examined 'in situ' all the blood vessels appeared to have joined the transplant peripherally; none was seen connecting directly with the central part. Hence restoration of blood supply occurred preferentially at the raw surface on the periphery of the transplant, and the smooth muscle near the serosal surface must have been nourished only by a plasma circulation for several days following transplantation.

The extent of the submucosal swelling in the transplant, reflected the loose attachment between mucosa and muscle coat in the guinea pig bladder wall. These layers are readily separated by oedema in the submucosa. Some workers (Makin, 1962; Menon et al, 1974) have utilized this fact in order to separate bladder mucosa from the muscle coat, by injecting saline into the submucosa.

In the present investigation the swelling in the submucosa was sub-divided by folds of mucosa. When the mucosal surface of the transplant was viewed in the S.E.M. at Day 6, these submucosal swellings appeared as three or four roughly hemispherical areas, separated by clefts. This arrangement was probably due to firmer adhesion between the mucosa and muscle coat at the base of the clefts.

The transplant epithelium, like that of skin grafts, lost its superficial cells soon after operation. A relatively normal epithelial thickness was recovered only when the nutrition to the underlying tissues was improved.

Hyaline material

The changes observed in the lamina propria of the transplant /

transplant were of two types; in the first, the cells and fibres of the lamina propria were lost and eventually replaced by new fibrous tissue: in the second, only the cells were lost leaving the collagen matrix which by Day 8 contained an accumulation of homogeneous hyaline material.

A similar hyaline material has been described beneath the cyst lining epithelium by Makin (1962), Kagawa (1965) and Menon et al (1974). Kagawa stated that this material was first observed at Day 7, whereas Makin reported its presence from Day 10 onwards, and Menon et al from Day 25 onwards. Makin (1962) suggested that this material was secreted by the epithelium, and this seems the most likely source, since the material appeared in sub-epithelial areas of necrotic fibrous tissue.

However, these authors all considered that the hyaline material was produced only by newly-formed epithelium, an opinion which gains no support from the present study where hyaline material could be found as early as Day 8. In the studies of Makin, Kagawa, and Menon et al, quoted above, only mucosa was transplanted, as opposed to whole bladder wall used in the present investigation. Their mucosal transplants prepared by the method of Makin (1962), were bound to consist of both epithelium and its supporting lamina propria (vide p.93). The presence of any lamina propria was ignored by these authors. In the present investigation hyaline material was formed in the lamina propria /

propria of the transplants and it seems reasonable to expect the formation of such material in the lamina propria beneath the original transplant epithelium in the studies of Makin (1962), Kagawa (1965) and Menon et al (1974). Furthermore those authors were not able to differentiate accurately between the positions of original transplanted and newly-formed epithelium. In the present study original transplanted epithelium was readily identified by the presence of smooth muscle beneath it (Fig. 66): in no case was hyaline material observed beneath epithelium which could be reliably identified as newly-formed. Indeed, the hyaline material demonstrated by Kagawa in his illustrations had a similar appearance to that observed only beneath original transplanted epithelium, in this investigation.

TRANSPLANT SITE

By Day 2, a fibrin clot had formed on the surface of the site tissues. This was progressively organized and replaced by fibrous connective tissue during the next eight days. Simultaneously the site tissues were overgrown by epithelium from the margin of the transplant.

McMinn (1969) and Wong & Martin (1977a) reported similar findings in healing experimental ulcers of the bladder wall, in cats and guinea pigs.

EPITHELIUM

Following transplantation, the whole of the transplant epithelium at first became reduced in thickness. This was due to a loss of surface cells, possibly as a result /

result of an inadequate blood supply. Similar effects have been described in whole thickness skin grafts (Davis & Traut, 1925; Mir y Mir, 1951). The epithelium close to the periphery rapidly regained a normal appearance as the blood supply was restored. Towards the centre of the transplant, although at one stage the epithelium was reduced to one cell in thickness, no breaks in its continuity were seen. Recovery here was slower, and only by Day 10 did the epithelium begin to approach a normal appearance.

During the first 12 days the epithelium of the transplant was elevated by the submucosal swelling, and the surface became smooth with only a few narrow folds. After resolution of the submucosal oedema, no folds remained (Figs. 43, 44, 45). The reduction in cyst perimeter total (C.P.T.) during this early period was probably not caused by a change in the area of the cyst floor/transplant site, since the transplant edges were firmly sutured in place. The magnitude of the reduction in C.P.T. suggests that the 'smoothing' of the transplant epithelium was accompanied by a reduction in its surface area.

The new epithelial growth at the edges of the transplant began to spread across the site tissues from Day 2 and was initially only one cell deep but became thicker by Day 6, completely covering the site tissues 8.35 days after operation.

Friedenstein (1961) in a study of bladder mucosal homotransplants /

homotransplants described two patterns of epithelial differentiation in the wall of the developing cyst. The first type developed an appearance similar to 'in situ' epithelium with its three distinct cell types. The second produced a tissue containing only one cell type, similar to that normally found only in the intermediate layer of in situ' epithelium. These two forms could also be distinguished by histochemical analysis of glycogen distribution. Friedenstein described a maximal glycogen concentration in the surface cells of the normally differentiated first type, but a uniform distribution in the second type of epithelium.

In the present investigation two such epithelial forms could not be distinguished. The epithelium appeared similar to 'in situ' epithelium when of approximately normal thickness though the superficial cells often appeared smaller. Up to Day 12 no difference in glycogen distribution could be observed. However, specimens in the present research were fixed in neutral buffered formalin, which according to Trott (1961b) would cause some loss of glycogen from the tissue if used for more than 24 hours. Since specimens were fixed for at least 48 hours, some loss of glycogen might be expected. Decalcification also results in a loss of tissue glycogen (vide p.109) and therefore glycogen distribution was examined only up to Day 12 in the present study. Thus it was not possible, with the light microscope, to confirm or deny the two patterns of epithelial /

epithelial differentiation observed by Friedenstein.*

Friedenstein (1961) employed his classification of the two types of epithelium to explain the presence or absence of bone in the cyst wall. He stated that bone formation was found only beneath epithelium in which all the cells were of one type. In a later study, which employed urinary bladder epithelium sealed into diffusion chambers, Friedenstein (1962) noted that bone formation had taken place despite the fact that the epithelium within the chambers retained its original (3-cell types) morphology. This evidence was presented without comment, despite the contradiction between it and his previous hypothesis.

Other workers did not usually distinguish between areas of epithelium on the grounds of histological appearance or histochemical analysis, although Makin (1962) and Huggins (1969) tried to distinguish between the positions of original transplanted, and newly-formed epithelium.

Epithelial hyperplasia

Areas of epithelial hyperplasia were observed from Day 6 onwards, essentially on the floor of the developing cyst. Only rarely were such areas seen on the cyst roof.

Mildly hyperplastic epithelium (Hyperplasia A) was found on the developing cyst floor from Day 6. Such hyperplasia was found some distance behind the epithelial edge, but subsequently appeared to spread progressively towards /

* Friedenstein used a special fixative for glycogen but took no account of whether his tissue was decalcified or not.

towards the floor centre. After the cyst floor covering was complete the outer-limit of the hyperplasia also moved towards the floor centre, leaving a smooth surface behind it. By Day 25 very little Hyperplasia A remained. Thus it would appear that Hyperplasia A was a transient phase of epithelial proliferation in the newly-formed epithelium of the cyst floor.

Papillomatous epithelial hyperplasia (Hyperplasia B) was first found at Day 14, within the remaining areas of Hyperplasia A near the centre of the floor. Hyperplasia B could still be observed at Day 180.

In a study of experimental ulceration of the bladder wall, Wong & Martin (1977a) described epithelial hyperplasias similar to those reported in the present investigation. Mild hyperplasia (similar to Hyperplasia A), with shedding of bulbous surface cells and epithelial thickening was seen from Day 2-6 in the new growth over the ulcer bed. Between Day 4 and 7 a ridge of hyperplastic epithelium (similar to Hyperplasia B) was found near the centre of the healed ulcer. In the present study, both forms of hyperplasia appeared later and lasted for a longer period than in the study of Wong & Martin. The later appearance was probably due to the absence of growth immediately following transplantation. The greater duration of the hyperplasias, especially Hyperplasia B may have been related to the proximity of bone, or possibly to the presence of cyst fluid instead of /

of urine over the epithelium.

Epithelial maturation

When examined with the S.E.M. the membrane of the surface epithelial cells in the developing cyst differed from those of bladder epithelium 'in situ'. Bladder epithelium 'in situ' was characterised by a cell surface covered by many small ridges which gave the membrane a crumpled appearance, while cyst floor epithelium, at Day 6, showed a cell membrane which was flat and dotted with many short microvilli. However, by Day 30 the surface of each cell possessed a typical crumpled appearance. A similar sequence of changes occurred in the cyst roof epithelium.

These results agree with those of Wong & Martin (1977a, b) who described the pattern of cell membrane changes following experimental bladder ulceration, and considered microvilli on a smooth cell membrane to be an immature pattern, and the acquisition of a crumpled cell membrane a sign of maturation. The findings of the present study would support this hypothesis.

In the cyst floor epithelium a pattern of maturation was observed with the T.E.M. between Day 10 and Day 40. At Day 10 the cells in all parts of the epithelium resembled those found normally only in the intermediate layer of 'in situ' bladder epithelium, and the luminal surface was characterised by many short microvilli. By Day 20 a differentiation towards three cell types was evident within the epithelium, and the luminal cell membrane /

membrane was like that of '<u>in situ</u>'epithelium, the differentiation becoming complete at Day 40.

Abdin & Friedenstein (1972) described the appearance of epithelium in non-inductive areas of the cyst wall up to Day 30. This contained just one cell type similar to that normally found only in the intermediate layer of bladder epithelium '<u>in situ</u>'. They also stated that 'as a rule' no basal lamina was found beneath bone-inducing epithelium, yet in their illustrations there appeared to be a basal lamina beneath such epithelium. The description of non-inductive epithelium in the above study completely contradicts that given by Friedenstein (1961, 1968) in light-microscope studies, and yet this difference is not mentioned by the authors.

The observations of the present study do not correspond with those of Abdin & Friedenstein. A well-defined basal lamina was usually observed beneath the epithelium (though when sectioned obliquely the lamina at times became difficult to discern). After Day 20 differentiation towards an '<u>in situ</u>' appearance was seen in all areas of the floor epithelium. The findings of Wong & Martin (1977a, b) and the present research indicate that as the newly-formed epithelium matured, a pattern could be observed, whereby changes of the luminal cell membrane were accompanied by a differentiation within the epithelium from one cell type only, to the three cell types characteristic of normal '<u>in situ</u>' epithelium. Friedenstein's (1961, 1968) and Abdin & Friedenstein's (1972) /

(1972) suggestion that the undifferentiated epithelium followed a path of differentiation distinct from that of normal bladder epithelium cannot be upheld in the light of the recent work, the epithelial form in question marking only a transient stage in the normal differentiation of newly-formed urinary epithelium.

OUTGROWTH EPITHELIUM

Areas of submerged epithelium were found from Day 4 onwards in the connective tissues of the transplant site. These areas appeared to be the result of proliferation into the site tissues of the new epithelial growth. The outgrowths were found at all levels within the fibrous connective tissue of the early cyst floor, but did not extend into the site skeletal muscle. Similar, though smaller and less extensive outgrowths were also seen in the cyst roof from Day 10 and from Day 6 at the interface between the edge of the transplant and the tissues site.

The S.A.T. of outgrowth epithelium per unit C.P.T. was maximal at Day 10 (Fig. 149) and declined thereafter; the break-up of the outgrowth epithelial strands into isolated epithelial islands by Day 25 could partly explain this decline.

In studies of the human urinary tract, sub-epithelial outgrowths, similar to those found in the present investigation, have been described by many workers. Von Brunn (1893) suggested that they arose in reaction to an inflammatory stimulus. Fagerstrom (1948) in an extensive analysis /

analysis of autopsy material, reported the presence of sub-epithelial masses in most of the cases examined from 20 years of age upwards. He found outgrowths in the form of crypts and solid cords of cells which were always connected to the surface epithelium. Johnson (1957) in a similar study found epithelial aberrancy in all cases over 15 years of age. He also examined the urinary tract of cows and sheep and found a similar occurrence of outgrowths in the form of crypts and islands of cells; again these were not associated with inflammatory processes.

In experimental animals, results are more variable. Crypt-like outgrowths were found by Johnson & McMinn (1955) in the healing of experimental ulcers of the cat bladder, but no solid epithelial cords or cell islands were found and neither was there evidence of associated inflammatory processes. Wong & Martin (1977a) in a similar study in the guinea pig, did not report the finding of any epithelial outgrowths.

McMinn & Johnson (1955) in a study of bladder mucosa transplanted to the rectus sheath in the cat, found similar crypt-like outgrowths. In the guinea pig, Friedenstein (1961) observed solid outgrowths beneath new epithelial growth, like those found in the present study. He reported that they were particularly marked at Day 10. Menon et al (1974) also mentioned the presence of outgrowths in their study, but gave no details. Friedenstein (1968) classified these epithelial outgrowths as 'atypical'; nonetheless their occurrence in normal or transplanted urinary tract appears to be a widespread phenomenon. /

phenomenon.

The actual form of the outgrowths appears to vary between species: only crypt-like outgrowths were found in the cat, whereas in the present research in the guinea pig most outgrowths were of the cord or island type.

CYST ENLARGEMENT

Prior to Day 10 only small amounts of smooth muscle were found beneath the edge of the cyst floor. Thereafter the proportion of cyst floor underlain by smooth muscle steadily increased (Fig. 153), possibly due to a proliferation of smooth muscle, or the incorporation of smooth muscle containing cyst wall into the floor. Proliferation could eventually lead to a cyst completely surrounded by smooth muscle, although no such observation was made up to Day 180, and therefore incorporation of wall into floor seemed more likely. An analysis of the map measurements showed that the area of cyst floor not underlain by smooth muscle did not change significantly up to Day 180. This supports the suggestion that the wall became incorporated into the cyst floor as the cavity enlarged. Apparently little or no movement of the transplant edge on the site tissues occurred.

If this was so, areas of the cyst floor which were originally part of the transplant could be distinguished by the presence of smooth muscle beneath them. Therefore the measurements of floor area underlain by neither bone nor smooth muscle (Neither F.A. Fig. 154) provided an indication /

indication of how little of the original transplant site did not become occupied by bone.

CHAPTER 7

DISCUSSION - BONE

BONE FORMATION RELATED TO CYST FLOOR EPITHELIUM

Early bone formation

The first signs of bone formation in the present study were found at Day 10 beneath newly-formed epithelium of the cyst floor. Areas of osteogenesis were rarely close to epithelial outgrowths.

Friedenstein (1961) also described initial bone formation at Day 10. He found this only beneath areas of undifferentiated epithelium which exhibited "infiltrative growth". This author considered the breakdown of the outgrowth epithelium to be a significant factor in osteogenesis. No other author has supported this view. The work of Johnson (1957) suggests that while epithelial outgrowth was to be expected, its disintegration, observed by Friedenstein, was atypical. Friedenstein used homotransplants, and it is possible that the epithelial breakdown he described resulted from epithelial rejection.

Makin (1962), Kagawa (1965), and Menon *et al* (1974) have described the earliest bone formation as occurring in areas of "pericystic hyaline formation", between Day 15 and Day 25. In the present investigation also, bone formation was found in areas of hyaline material.

A few small isolated foci of osteogenesis were found beneath areas of hyaline material from Day 17 onwards, (i.e. /

(i.e. seven days after the onset of bone formation in the cyst floor). However, in most cases it could be shown that the bone formation had commenced in the cyst floor (where no hyaline material was found) and only later spread into areas containing hyaline.

Furthermore the results of the present research, discussed earlier (vide p. 94) suggest that if an area of epithelium was underlain by hyaline material, this epithelium was part of the original transplant and was not new growth. Huggins (1969) stated that bone formation only occurred beneath new epithelial growth, and in the present investigation the majority of bone found was also related to new epithelial growth.

Observations of initial ossification in areas of hyaline material, as described by Makin (1962), Kagawa (1965) and Menon et al (1974), are at variance with this evidence. It is probable that the first bone formation in these three studies occurred in non-hyaline areas, but rapidly spread into surrounding areas where the hyaline material was found. Again, Makin (1962) states that this material was only found in part of the cyst wall. Such confusion could easily have arisen since in the studies of Makin (1962) Kagawa (1965) and Menon et al (1974) the transplant was simply placed in a tissue pocket and was not carefully orientated and fixed in position. In the present research the smooth muscle of the transplant was used as a marker to ensure accurate identification of the /

the original epithelium. It is noteworthy that in the work of other authors no reference was made to the possible preosseous role of the hyaline material.

Bone formation generally commenced later in the studies of Makin (1962), Kagawa (1965) and Menon et al (1974) than in the present investigation and those of Leowi (1954) and Friedenstein (1961). This delay may have been due to trauma to the epithelium during its separation from the muscle coat, which could have retarded epithelial proliferation.

The early bone had the appearance of woven bone, the present investigation agreeing with Makin (1962) and Menon et al (1974). However, when examined in polarized light the 'criss-cross' pattern of collagen fibres in the early bone described by Menon et al (1974) was not observed, the fibres in the bone running predominantly parallel to the cyst floor from Day 12 onwards. Thus the present results would suggest that although the early bone had an immature appearance, the pattern of fibres within it was much more like that of mature bone.

When the new bone was stained by the P.A.S. technique it was strongly positive, confirming the results of earlier workers (Leowi, 1954; Makin, 1962; Menon et al, 1974). A difference was noted between decalcified and undecalcified sections. The bone in the undecalcified sections was strongly P.A.S.-positive and amylase-resistant, whereas that in the decalcified sections was P.A.S.-positive but not

not amylase-resistant. Also the fine P.A.S.-positive amylase-resistant granules were not seen in decalcified sections. This difference suggests that the bone mineral had associated with it a P.A.S.-positive amylase-resistant component which on decalcification was lost or became P.A.S-negative.

The P.A.S.-positive material found after decalcification was almost certain to be glycogen since it was not amylase resistant. However, Kugler & Wilkinson (1961) and Trott (1961a) have shown that the glycogen which is histochemically detected by the P.A.S. technique is acid soluble, and is totally removed following seven days treatment in a decalcifying solution. In the present investigation, specimens up to and including Day 10 were not decalcified. Some at Day 12 and all from Day 14 onwards were decalcified. The early specimens (i.e. up to Day 40) were decalcified for two days only*, and so the P.A.S. positive material observed in these specimens was glycogen which might have been relocated. For this reason the results obtained from decalcified material stained by the P.A.S. technique were considered unreliable and therefore discarded.

Matrix Vesicles

In this investigation T.E.M. studies of osteogenetic areas showed that the earliest crystals found were in small vesicles (Type 2 vesicles), usually spherical, lying in intercellular seams of presumptive osteoid. The /

* (The Day 100 and 180 specimens were decalcified for six days)

The crystals grew within these vesicles and apparently ruptured the vesicular wall to form a nucleus for an apatite cluster. These clusters enlarged to well over 1 µm in diameter before eventually fusing into seams of bone. It is probable that these apatite clusters were the structures identified by light microscopy as P.A.S-positive amylase-resistant granules, i.e. they were of a similar size, and were also found in the same site at a corresponding time. Furthermore these structures were not seen after decalcification which would be expected if they were actually apatite clusters.

The only previous ultrastructural study of bladder epithelium-induced osteogenesis (Abdin & Friedenstein, 1972) did not report early apatite crystallization within extra-cellular vesicles, nor the relationship between apatite clusters and P.A.S-positive granules.

The Type 2 vesicles described in the bone matrix in this study were similar to structures first described by Anderson (1967) in experimentally induced calcifying cartilage. These vesicles have since acquired the name 'matrix vesicles' and have been reported in early membrane bone formation '<u>in vivo</u>' by Bernard & Pease (1969), in early membrane bone formation '<u>in vitro</u>' by Marsavo & Bernard (1977), and in many other diverse sites of early tissue calcification (Anderson, 1976).

Matrix vesicles are usually 0.1-0.2 µm in diameter and are enveloped by a trilaminar membrane (Anderson, 1976). This corresponds well with the Type 2 vesicles described /

described in this study (Figs. 101, 105). Bonucci (1970) has reported that matrix vesicles, which are large enough to be seen in the light microscope are P.A.S-positive. However, the matrix vesicles observed in this present study were too small to be seen in the light microscope. In a review article, Anderson (1976) stated that divergent views have been expressed as to the origin of matrix vesicles. Some authors have suggested that these vesicles arise as buds from cell processes. Other authors propose that vesicles are assembled extra-cellularly from sub-microscopic precursors. No evidence was found in the present study for or against either view.

In a recent study of early membranous ossification 'in vitro', Marsavo & Bernard (1977) described five sequential stages -

(a) Differentiation of osteoblasts from mesenchymal cells.

(b) The appearance of matrix vesicles in the extracellular matrix.

(c) The formation of crystals in these vesicles.

(d) The growth of the crystals to form an apatite nodule or cluster.

(e) The coalescence of these nodules to form seams of bone.

All these stages were observed in the present study, and therefore the bone formation in the cyst floor fulfilled the /

the criteria for membranous bone formation ('in vivo') according to Bernard & Pease (1969).

Growth of the bony plaque

By Day 17 the bone in the cyst floor had come to occupy most of the area free of smooth muscle tissue and thereafter little further spread of bone occurred. When the section and map measurements were analysed all the bony plaque parameters showed marked increases up to Day 17. During the following eight days no significant changes took place. This initial period of rapid growth followed by a period of minimal growth has not been reported previously. The period of minimal growth may represent a quiescent phase in bone formation, or an exaggeration of an insignificant change, due to the small numbers in each group of animals.

At Day 17 (i.e. at the beginning of the minimal bone growth period) osteoclasts were first seen on the surface of the bone spaces. Reversal lines also became apparent within the bone structure from this time. Therefore remodelling rather than growth or extension of the plaque took place between Day 17 and Day 25. Such remodelling could have been a prerequisite for the increase in thickness which took place after Day 25.

Although the area of cyst floor underlain by bone increased very little (10%) after Day 25, the mean bone thickness increased steadily up to Day 180 (by a total of 316%). No statistically significant change in the Floor Bone S.A.T/C.P.T. ratio, after Day 25 could be detected.

The /

The increase in bone thickness up to Day 40 appeared to be due to appositional growth on both surfaces parallel to the cyst floor.

At Day 40 on the central part of the plaque surface, facing the epithelium, a thick layer of osteoid formed, and here bone growth continued up to Day 180. On the remaining parts of the plaque surface however, no osteoid could be detected under the light microscope, and it appeared that these surfaces had stopped growing. However, when one of these "non-growing" parts of the plaque surface was examined with the T.E.M. a narrow band of collagenous matrix was seen between the bone surface and an incomplete layer of osteoblasts. In this uncalcified matrix, no Type 2 vesicles (matrix vesicles) were found, but Type 1 vesicles (cell processes) were clearly seen. The absence of matrix vesicles in such an area correlated well with the cessation of bone growth assessed from the light microscope.

From Day 40 onwards the bone of the plaque was separated from the underlying striated muscle by a narrow layer of fibrous tissue only. This layer appeared to be of similar thickness to that found covering the striated muscle (i.e. epimysium) in the Day 0 specimens. This suggested that bone formation had extended to the limit of the new fibrous tissue of the cyst floor, but could not penetrate into the original fibrous tissue of the epimysium beyond.

The edges of the plaque by Day 40 had come to lie close /

close to or slightly overlapping the edge of the smooth muscle beneath the peripheral cyst floor. The analysis of the map measurements indicated that the peripheral part of the cyst floor (underlain by smooth muscle) was directly derived from the transplant. This suggested that the peripheral spread of the bone was again limited to the new fibrous tissue of the transplant site. However, occasionally extensions from the margin of the plaque up into the cyst wall were found (this phenomenon is discussed on p. 117).

Friedenstein (1961) reported between Day 20 and 24 the appearance of hyaline cartilage within the bone. He considered this cartilage analagous to the secondary cartilage found during the development of membrane bones. Makin (1962) and Menon et al (1974) found no cartilage associated with the bone formation around their cysts. Neither was cartilage seen in the present investigation, though some extensive areas of osteoid were observed at a similar stage to that reported by Friedenstein. The illustration of Friedenstein's cartilage shows some similarity to the areas of osteoid seen in the present investigation.

Most workers do not comment on changes in the bone after Day 40. Makin (1962) stated that bone growth ceased around Day 40, and that the bone persisted for about six months. The bone growth observed in this present study between Day 40 and Day 180 has not been previously reported.

In /

In the present investigation after Day 40 there was a loss of epithelium overlying the thick layer of osteoid at the surface of the bony plaque. The margin of this denuded area was marked by a ring of papillomatous epithelial hyperplasia. In spite of the absence of epithelium, bone formation continued only in such areas after Day 40.

The degree of dependence of bone formation upon the epithelium which induced it, is not generally agreed. Friedenstein (1961) stated that bone which had formed in relation to homogenous epithelium was resorbed after the epithelium had been destroyed. However, Zaleski (1961), in a similar experiment, was able to demonstrate vital bone tissue 120 days after the destruction of the epithelium. The continued bone growth in areas apparently denuded of epithelium, as reported in this present study is in harmony with the findings of Zaleski.

Spaces were seen in the cyst floor bone from Day 12 onwards. Initially they appeared to be orientated roughly perpendicular to the surface of the floor. As the spaces enlarged and coalesced they became more parallel to the floor, especially within the core of the bony plaque. From Day 40 onwards the centre of the plaque was occupied by an extensive space containing haemopoietic tissue. Within the bone, typical multi-nucleate osteoclasts were observed from Day 17. The presence of osteoclasts within the induced bone has been /

been reported previously only by Beresford & Hancox (1967) who gave no further details, and Makin (1962) stated that no osteoclasts were observed in his study. In the present study the number of osteoclasts per unit bone spaces surface area was analysed between Day 17 and Day 40, but no statistically significant change could be detected. (This may have been due to the large sampling interval, and the non-uniform distribution of these cells within the bone).

Recent work reviewed by Owen (1978) has shown that osteoclasts develop from a cell line histogenetically distinct from that which gives rise to osteoblasts. Osteoclasts are derived from the haemopoietic cell line found in bone marrow (via precursor cells, monocytes or macrophages). Cross-circulation studies in rats (Gothlin & Ericsson, 1976) have shown that circulating precursor cells from one animal can give rise to osteoclasts in another. It would appear that the first osteoclasts found in the present study are also developed from circulating precursors, since haemopoietic marrow does not develop locally until 13 days later.

BONE FORMATION RELATED TO OUTGROWTH EPITHELIUM

Bone formation was found related to outgrowth epithelium from Day 13 onwards. (Though in relation to the small outgrowths beneath the roof epithelium no bone was observed). The early stages of this bone formation closely resembled those seen in the cyst floor. The S.A.T. of this bone was always considerably less than the corresponding /

corresponding Floor Bone S.A.T. for the bone in the cyst floor (Fig. 146). This was in accord with the much smaller volume of outgrowth epithelium compared to that of cyst floor epithelium.

Some areas of outgrowth-related bone were incorporated into the spreading bony plaque; the remaining areas of bone appeared to increase in size up to Day 25, but after Day 25 appeared to reduce in size at a slower rate. When the section measurements were analysed there was an apparently confirmatory rise in the S.A.T. of outgrowth related bone up to Day 25, but no significant change was found after Day 25. These results must however be interpreted with caution (vide appendix A).

BONE FORMATION RELATED TO CYST ROOF EPITHELIUM

Most of these areas of bone formation were extensions from that found in the cyst floor. Few completely isolated areas of bone formation were found deep to the roof epithelium. None of these areas was related to outgrowths from the cyst roof epithelium. The S.A.T. of this bone in the cyst roof was always very small in comparison with that in the cyst floor or that related to outgrowths (Fig. 146).

The bone formation appeared to commence beneath the hyaline material and only later to spread into it. This would be expected, since the hyaline zone rarely contained cells, and so the nearest inducible cells to the epithelium would be in the sub-hyaline connective tissue. The separation of the inducing epithelium and the reacting cells, by /

by a layer of hyaline material, might suggest that the inductive process was a humoral rather than a contact phenomenon. The humoral nature of the inductive activity of bladder epithelium has been previously reported by Friedenstein (1962) in a study using millipore diffusion chambers.

The formation of areas of bone remote from the cyst lumen, related to outgrowths, yet apparently similar to cyst floor bone, while there was very little bone formation beneath cyst roof epithelium, would suggest that cyst fluid played no significant role in the induction and formation of bone.

Many workers have considered that only new growth of bladder epithelium is able to induce bone formation, and that this induction can only occur in non-bladder connective tissues, e.g. Huggins (1969). However, Beresford & Hancox (1967) and Popovic et al (1967) have both reported bone formation in the guinea pig bladder wall following injury. Here new epithelial growth might be expected but the connective tissue would still be that of bladder wall which in most species will not respond to an osteo-inductive stimulus. The issue is perhaps still in doubt in the guinea pig and warrants further study. The results of the present investigation would appear to support the findings of Beresford & Hancox (1967) and Popovic et al (1967).

CHAPTER 8

CYST FLUID

RESULTS

MACROSCOPIC APPEARANCE

From Day 8 onwards fluid was found within the cyst cavity when its roof was incised. The fluid was watery in consistency and varied in colour from pale yellow to murky brown. It was always under pressure and there appeared to be a steady increase in the volume of fluid over the experimental period.

MICROSCOPIC APPEARANCE

The cyst contents were seen as a shrunken eosinophilic mass, containing red and white blood cells, epithelial cells, and at times some crystals. White blood cells were particularly common in the cyst fluid up to Day 12, the number subsiding thereafter. Many red blood corpuscles were usually seen in the fluid, their quantity varying considerably from one cyst to another. Exfoliated epithelial cells were normally present in the cyst fluid, often in clumps; although some of the cells had a necrotic appearance, many did not (Figs. 155, 156).

Crystals

Crystals were found in about a quarter of the cysts examined between Day 10 and Day 100. The crystals were usually lying free in the fluid (Figs. 155, 156). Some were also found in the lumen of accessory cysts (Fig. 157), and even in the core of epithelial outgrowths (Fig. 158). These /

These crystals were found whether the specimen was processed for light microscopy, T.E.M. or S.E.M; they were also retrieved from a freshly opened cyst cavity. The crystals were red-brown in colour in unstained paraffin sections, and of the stains routinely used, were only stained by picric acid. Decalcification did not alter the appearance of the crystals. They were less hard than woven bone, since in undecalcified sections the bone shattered, but the crystals did not. They were usually cut cleanly with little displacement from their original position.

There was no discernible correlation between any cyst feature, and the presence or absence of crystals.

SCANNING ELECTRON MICROSCOPIC APPEARANCE

Crystals observed in the scanning electron microscope at Day 30, were found to be tetrahedral in shape, often truncated with a maximum side length of 200µm. The crystals were usually partially coated with red blood corpuscles. (Figs. 159, 160).

DISCUSSION

The finding of crystals in the cyst lumen was quite unexpected. Apart from Makin (1962) who mentioned the finding of minute calculi, but gave no further details, the finding of such crystals has not been previously reported. The crystals probably had an organic composition, since they sectioned easily and were apparently unaffected by decalcification.

Alvarez- /

Alvarez-Ierena (1952), in a study of sealed dogs' bladders from which the urine had been diverted, found many small crystals in the bladder lumen. These crystals were elongated, brown in colour, and measured 150µm long and 10µm wide.

The crystals found in the present study were of a different shape, perhaps the result of the species difference. However, in both studies the crystals were found in tissue cavities lined by urinary bladder epithelium, which might suggest an origin for the crystals, from an epithelial secretion or transudate. Further study of this phenomenon would seem to be required.

CHAPTER 9

FINAL COMMENTS

BONE FORMATION

This thesis has shown that membrane bone formation regularly occurred ten days after the transplantation of autogenous guinea pig urinary bladder to a soft tissue site. The bone formation usually occurred close to new epithelial growth and was rapid during the first seven days, followed by a less active period during which remodelling took place. The bone seemed to be limited to the new fibrous tissue, which developed at the transplant site. Many of the histological and ultrastructural features of embryonic membrane bone formation were seen, despite the fact that it was occurring in a young adult animal. The new bone was still present six months after transplantation.

Heterotopic osteogenesis of this type would seem to be a useful experimental model. As the time and position of the initial bone formation can be predicted accurately, this makes it possible to study the development of bone cells, prior to the onset of osteogenesis. The effects of diet or drugs, or the influence of genetic factors on the early bone formation are other possible fields of study. This experimental model is also suitable for the investigation of possible treatments for clinical conditions such as myositis ossificans progressiva.

Clinical applications of urinary epithelium induced osteogenesis /

osteogenesis are at present limited by the necessity for vital urinary epithelium. This problem might be overcome by the use of cultured cell lines. One such dental application would be in the repair of the bone destruction which is a major cause of tooth loss in periodontal disease. The active inductive agent in the osteogenic process has not yet been identified; Friedenstein (1962) has suggested it may be humoral in nature. If this principle could be isolated the prospects for a clinical application would indeed be promising.

QUANTIFICATION

The statement by Lord Kelvin quoted at the beginning of this thesis highlights the importance of measurement as opposed to pure observation. In this thesis, the measurements often provided only confirmatory evidence for the observational impressions. However, due to the greater potential accuracy and sensitivity of the measurements, it was only following an analysis of these measurements that the phase of reduced bone growth (Day 17-25) was detected and the apparent extension of smooth muscle from the transplant into the tissue beneath the cyst floor was found to be unsubstantiated.

APPENDIX A

EXPERIMENTAL VARIATION

FIXATION AND PROCESSING OF TISSUE

The tissue was processed through a standardized regime. The material was subject to shrinkage of 50% for intact cysts, but a large proportion of this was due to the reduction in cyst fluid volume during dehydration. This shrinkage was taken to be consistent over the experimental series.

SECTION THICKNESS AND SAMPLING INTERVAL

These factors were only pertinent to the analysis of the morphometric measurements. When tested with a dial micrometer, the microtomes proved highly accurate and consistent and so a uniform thickness was assumed for the series.

The measurement of sections at 500 µm intervals was chosen as the best compromise between accuracy and economic use of resources. The error due to sampling is inversely proportional to the square root of the number of measurements, so that a large increase in the number of measurements would be necessary to decrease the errors significantly.

The accuracy with which a given sampling technique will estimate the true values, can be examined by cumulative mean plots (Williams, 1977). A series of such plots were constructed from one specimen of Day 25 for - Floor Bone S.A.T., Outgrowth S.A.T., Outgrowth Bone

Bone S.A.T., and Roof Bone S.A.T. Mean values for each parameter were plotted for measurements made at sampling intervals of 2,000; 1,000; 500 and 100 μm. The means obtained by sampling at 100 μm were taken as true values for purposes of comparison.

Floor Bone S.A.T. was within 10% of the true value at 2,000 μm sampling interval, whereas Outgrowth S.A.T. was only within 10% at a 500 μm sampling interval. Both Outgrowth Bone S.A.T. and Roof Bone S.A.T. were not within 10% at a 500 μm sampling interval, probably due to their relative sparsity, and non-homogeneous distribution. These two parameters were therefore not satisfactorily estimated for this specimen. However, when Outgrowth Bone S.A.T. was examined for many specimens a significant trend was found, which would suggest that a 500 μm sampling interval could be adequate if a number of specimens are examined. No such trend was found for Roof Bone S.A.T. The effect of sampling on the cyst floor maps was similar to that described for Floor Bone S.A.T.

APPENDIX B

MATHEMATICS & STATISTICS

Each set of measurements of one cyst was totalled, corrected for magnification, and tabulated (vide Tables B and C). The means and standard deviations of the Cyst lumen perimeter length totals and Cyst floor areas in each time group (e.g. Day 2) were calculated. The other totals were expressed as ratios (e.g. Outgrowth sectional area total/Cyst lumen perimeter length total). The means and standard deviations of the ratios in each time group were then computed and tabulated (vide Tables B and C). Ratios were used in order to compensate for the variation in cyst size.

Graphs were plotted of each parameter or ratio, against time. Each mean was indicated with an accompanying vertical bar, representing plus or minus one standard deviation. Linear regression lines were calculated for each graph and tested for significance. If a regression line for one time interval (e.g. Day 10-25) was not statistically significant the data from the adjacent time interval was included (e.g. Day 25-180) and a new regression line calculated and tested for significance.

The regression coefficients were tested for a significant difference from the hypothetical value zero, by the calculation of t (n was usually <30).

Formulae /

Formulae

for a straight regression line $y = a + bx$

(1) $$b = \frac{\sum(x - \bar{x})\,(y - \bar{y})}{\sum(x - \bar{x})^2}$$

(2) $$a = \bar{y} - b\bar{x}$$

When $n < 30$

(3) $$t = \frac{b}{s \Big/ \sqrt{\sum(x - \bar{x})^2}}$$ Bailey (1968)

(t has n - 2 degrees of freedom)

where $$s^2 = \frac{1}{n - 2}\left[\sum(y - \bar{y})^2 - \frac{\left(\sum(x - \bar{x})\,(y - \bar{y})\right)^2}{\sum(x - \bar{x})^2}\right]$$

APPENDIX C

STEREOLOGY

Stereology is a method for obtaining three-dimensional quantitative information from two dimensional sections on the basis of geometrico-statistical reasoning. If the tissue being examined can be considered homogeneous, the unbiased data required for accurate application of this method can be obtained by systematic random sampling of the tissue (Weibel, 1969). Systematic random sampling has been shown to give superior results over other methods of sampling (Ebbesson & Tang, 1967). This method uses samples taken at regular intervals throughout the tissue, the sample lattice being applied at random. Regularly spaced serial sections throughout a block of tissue, is one example.

Stereological relationships were only used in the study of bone spaces, since only they were considered to be homogeneously distributed within their bounding volume (bone). Two relationships were applied.

(1) $A_A = V_V$

That is, the areal fraction of a component (bone spaces) within the bounding area (bone) is on average equal to the volume fraction of that component within the bounding volume. This relationship was first derived by Delesse (1848) and has since been independently derived by several workers including Weibel (1963).

(2) /

(2) $$B_A = \frac{4}{\pi} S_V$$

That is the density of profile border length (spaces perimeter) on the bounding area (bone) is directly proportional to the density of surface area in the bounding volume. This relationship was first established by Saltykov (1958).

Therefore:

(1A) $$\frac{\text{Bone spaces S.A.T.}}{\text{Floor Bone S.A.T.}} = \frac{\text{Bone spaces Vol.}}{\text{Bone Vol.}}$$

(2A) $$\frac{\text{Bone spaces P.T.}}{\text{Floor Bone S.A.T.}} = \frac{\text{Bone spaces S.A.}}{\text{Bone Vol.}}$$

(3) $$\frac{\text{Bone spaces P.T.}}{\text{Bone spaces S.A.T.}} = \frac{\text{Bone spaces S.A.}}{\text{Bone spaces Vol.}}$$

DATA TABLES

TABLES - ABBREVIATIONS

C.P.T. - Cyst lumen perimeter length total

F.B.S.A.T.+ Bone sectional area total related to cyst floor epithelium, i.e. Floor Bone S.A.T.

Sp. Vol. - Bone spaces volume in the above bone

Sp. S.A. - Bone spaces surface area in the above bone

O.S.A.T. - Epithelial outgrowth sectional area total

O.B.S.A.T. - Bone sectional area total related to epithelial outgrowths, i.e. Outgrowth Bone S.A.T.

R.B.S.A.T. - Bone sectional area total related to cyst roof epithelium, i.e. Roof Bone S.A.T.

C.F.A. - Cyst floor area

E.F.A. - Epithelium covered cyst floor area

B.F.A. - Cyst floor area underlain by bone

C.F.A. * - Cyst floor area

S.F.A. * - Cyst floor area underlain by smooth muscle

O.F.A. * - Cyst floor area underlain by an overlap between smooth muscle and bone

N.F.A. * - Cyst floor area underlain by neither bone nor smooth muscle

M.T.B. - Mean sectional thickness of cyst floor bone

+ F.B.S.A.T. is equivalent to Bone Volume (Bo. Vol.) in Sp. Vol/Bo. Vol. ratio

* Van Gieson stained sections only

TABLE A

SECTION MEASUREMENTS

IMMEDIATE POST-OPERATIVE	1	2	3	MEAN	STD. DEV.
C.P.T.	3.545×10^{2}	3.685×10^{2}	1.997×10^{2}	3.076×10^{2}	9.368×10^{1}
C.F.A.	9.121×10^{1}	1.104×10^{2}	6.108×10^{1}		
E.F.A.	3.880×10^{1}	0.000	0.000		
E.F.A/C.F.A.	4.254×10^{-2}	0.000	0.000	1.418×10^{-2}	2.456×10^{-2}
TWO DAYS	71	96	54	MEAN	STD. DEV.
C.P.T.	2.281×10^{2}	1.987×10^{2}	2.191×10^{2}	2.153×10^{2}	1.506×10^{1}
O.S.A.T.	3.011×10^{-2}	1.400×10^{-3}	2.902×10^{-3}		
O.S.A.T/C.P.T*	2.397×10^{-4}	1.273×10^{-5}	2.268×10^{-5}	9.170×10^{-5}	1.000×10^{-4}
C.F.A.	1.032×10^{2}	9.060×10^{1}	9.290×10^{1}		
E.F.A.	7.000×10^{-1}	1.870	1.710		
E.F.A/C.F.A.	6.783×10^{-3}	2.064×10^{-2}	1.841×10^{-2}	1.528×10^{-2}	7.440×10^{-3}

* C.P.T. corrected for bare areas of cyst floor

FOUR DAYS	72	97	55	MEAN	STD. DEV.
C.P.T.	2.824×10^{2}	2.158×10^{2}	2.073×10^{2}	2.352×10^{2}	4.112×10^{1}
O.S.A.T.	3.744×10^{-1}	2.577×10^{-1}	1.390×10^{-1}		
O.S.A.T/C.P.T.*	1.756×10^{-3}	1.897×10^{-3}	1.048×10^{-3}	1.567×10^{-3}	4.359×10^{-4}
C.F.A.	1.041×10^{2}	9.712×10^{1}	8.347×10^{1}		
E.F.A.	3.490×10^{1}	1.718×10^{1}	8.780		
E.F.A/C.F.A.	3.352×10^{-1}	1.769×10^{-1}	1.052×10^{-1}	2.058×10^{-1}	1.77×10^{-1}

* C.P.T. corrected for bare areas of cyst floor

SIX DAYS	73	58	98	MEAN	STD. DEV.
C.P.T.	1.405×10^{2}	1.395×10^{2}	1.157×10^{2}	1.319×10^{2}	1.404×10^{1}
O.S.A.T.	5.350×10^{-1}	1.141	1.076		
O.S.A.T/C.P.T.*	3.974×10^{-3}	9.420×10^{-3}	1.029×10^{-2}	7.895×10^{-3}	3.423×10^{-3}
C.F.A.	3.596×10^{1}	5.759×10^{1}	4.373×10^{1}		
E.F.A.	3.008×10^{1}	3.912×10^{1}	2.675×10^{1}		
E.F.A/C.F.A.	8.365×10^{-1}	6.793×10^{-1}	6.117×10^{-1}	7.092×10^{-1}	1.153×10^{-1}

* C.P.T. corrected for bare areas of cyst floor

EIGHT DAYS	99	74	69	MEAN	STD. DEV.
C.P.T.	$1,249 \times 10^{2}$	1.409×10^{2}	1.051×10^{2}	1.236×10^{2}	1.793×10^{1}
O.S.A.T.	2.939×10^{-1}	9.300×10^{-1}	6.470×10^{-1}		
O.S.A.T/C.P.T.*	2.454×10^{-3}	8.116×10^{-3}	6.156×10^{-3}	5.575×10^{-3}	2.876×10^{-3}
C.F.A.	5.539×10^{1}	5.447×10^{1}	3.196×10^{1}		
E.F.A.	5.027×10^{1}	3.216×10^{1}	3.196×10^{1}		
E.F.A/C.F.A.	9.076×10^{-1}	5.904×10^{-1}	1.000	8.327×10^{-1}	2.146×10^{-1}

* C.P.T. corrected for bare areas of cyst floor

TEN DAYS	59	.75	87	MEAN	STD. DEV.
C.P.T.	8.598×10^{1}	1.390×10^{2}	1.115×10^{2}	1.222 x 102	2.652×10^{1}
F.B.S.A.T.	4.940×10^{-3}	2.005×10^{-1}	1.476×10^{-1}		
F.B.S.A.T/C.P.T.	5.740×10^{-5}	1.442×10^{-3}	1.324×10^{-3}	9.411×10^{-4}	7.670×10^{-4}
O.S.A.T.	5.706×10^{-1}	2.392	1.512		
O.S.A.T/C.P.T.	7.807×10^{-3}	1.728×10^{-2}	1.356×10^{-2}	1.288×10^{-2}	4.773×10^{-3}
O.B.S.A.T.	3.460×10^{-2}	8.650×10^{-3}	0.000		
O.B.S.A.T/O.S.A.T.	5.159×10^{-2}	3.616×10^{-3}	0.000	1.840×10^{-2}	2.879×10^{-2}
O.B.S.A.T/C.P.T.	4.024×10^{-4}	6.220×10^{-5}	0.000	1.548×10^{-4}	2.163×10^{-4}

TWELVE DAYS	88	.76	60	MEAN	STD. DEV.
C.P.T.	9.787×10^{1}	1.663×10^{2}	9.599×10^{1}	1.200×10^{2}	4.006×10^{1}
F.B.S.A.T.	7.651×10^{-1}	4.160×10^{-1}	6.503×10^{-1}		
F.B.S.A.T/C.P.T.	7.817×10^{-3}	2.501×10^{-3}	6.775×10^{-3}	5.698×10^{-3}	2.817×10^{-3}
Sp. Vol.	1.990×10^{-1}	1.196×10^{-1}	1.127×10^{-1}		
Sp. Vol/Bo. Vol.	2.601×10^{-1}	2.875×10^{-1}	1.733×10^{-1}	2.403×10^{-1}	5.962×10^{-2}
Sp. S.A.	1.486×10^{1}	8.336	9.923		
Sp. S.A/Sp. Vol.	7.467×10^{1}	6.970×10^{1}	8.805×10^{1}	7.747×10^{1}	9.491
O.S.A.T.	8.171×10^{-1}	5.206×10^{-1}	4.100×10^{-1}		
O.S.A.T/C.P.T.	8.349×10^{-3}	3.130×10^{-3}	4.271×10^{-3}	5.583×10^{-3}	3.310×10^{-3}
O.B.S.A.T.	1.073×10^{-1}	1.077×10^{-1}	3.183×10^{-2}		
O.B.S.A.T/O.S.A.T.	1.313×10^{-1}	2.069×10^{-1}	7.763×10^{-2}	1.386×10^{-1}	6.494×10^{-2}
O.B.S.A.T/C.P.T.	1.096×10^{-3}	6.476×10^{-4}	3.315×10^{-4}	6.917×10^{-4}	3.605×10^{-4}

FOURTEEN DAYS	77	53	89	MEAN	STD. DEV.
C.P.T.	5.868×10^{1}	2.014×10^{2}	1.275×10^{2}	1.292×10^{2}	7.137×10^{1}
F.B.S.A.T.	9.596×10^{-1}	3.756	4.899×10^{-1}		
F.B.S.A.T/C.P.T.	1.635×10^{-2}	1.865×10^{-2}	3.843×10^{-3}	1.295×10^{-2}	7.968×10^{-3}
Spaces Vol.	2.390×10^{-1}	1.000	7.181×10^{-2}		
Spaces Vol/Bone Vol.	2.491×10^{-1}	2.662×10^{-1}	1.466×10^{-1}	2.206×10^{-1}	6.468×10^{-2}
Spaces S.A.	1.417×10^{1}	4.876×10^{1}	7.581		
Spaces S.A/Vol.	5.929×10^{1}	4.876×10^{1}	1.056×10^{2}	7.122×10^{1}	3.024×10^{1}
O.S.A.T.	4.525×10^{-1}	2.476	2.530×10^{-1}		
O.S.A.T./C.P.T.	7.711×10^{-3}	1.229×10^{-2}	1.984×10^{-3}	7.328×10^{-3}	5.164×10^{-3}
O.B.S.A.T.	0.000	8.828×10^{-1}	0.000		
O.B.S.A.T/O.S.A.T.	0.000	3.665×10^{-1}	0.000	1.888×10^{-1}	2.038×10^{-1}
O.B.S.A.T/C.P.T.	0.000	4.283×10^{-3}	0.000	1.461×10^{-3}	2.530×10^{-4}

SEVENTEEN DAYS	95	107	108	MEAN	STD. DEV.
C.P.T.	1.185×10^{2}	7.358×10^{1}	1.706×10^{2}	1.209×10^{2}	4.855×10^{1}
F.B.S.A.T.	2.433	9.518×10^{-1}	7.291		
F.B.S.A.T/C.P.T.	2.053×10^{-2}	1.293×10^{-2}	4.275×10^{-2}	2.540×10^{-2}	1.549×10^{-2}
Spaces Vol.	7.438×10^{-1}	2.046×10^{-1}	2.103		
Spaces Vol/Bone Vol.	3.057×10^{-1}	2.150×10^{-1}	2.884×10^{-1}	2.697×10^{-1}	4.815×10^{-2}
Spaces S.A.	4.301×10^{1}	1.668×10^{1}	1.305×10^{2}		
Spaces S.A/Vol.	5.782×10^{1}	8.152×10^{1}	6.205×10^{1}	6.713×10^{1}	1.264×10^{1}
O.S.A.T.	1.713×10^{-1}	2.790×10^{-1}	3.141×10^{-1}		
O.S.A.T/C.P.T.	1.445×10^{-3}	3.792×10^{-3}	1.841×10^{-3}	2.359×10^{-3}	1.256×10^{-3}
O.B.S.A.T.	2.193×10^{-1}	8.566×10^{-2}	1.246×10^{-1}		
O.B.S.A.T/O.S.A.T.	1.280	3.070×10^{-1}	3.967×10^{-1}	6.612×10^{-1}	5.377×10^{-1}
O.B.S.A.T/C.P.T.	1.851×10^{-3}	1.164×10^{-3}	7.303×10^{-4}	1.248×10^{-3}	5.650×10^{-4}
R.B.S.A.T.	2.040×10^{-2}	0.000	3.729×10^{-2}		
R.B.S.A.T/C.P.T.	1.721×10^{-4}	0.000	2.185×10^{-4}	1.302×10^{-4}	1.148×10^{-4}

TWENTY DAYS	93	.63	94	MEAN	STD. DEV.
C.P.T.	1.122×10^{2}	1.168×10^{2}	1.110×10^{2}	1.133×10^{2}	3.061
F.B.S.A.T.	4.848	2.917	1.118		
F.B.S.A.T/C.P.T.	4.321×10^{-2}	2.496×10^{-2}	1.007×10^{-2}	2.608×10^{-2}	1.660×10^{-2}
Spaces Vol.	1.094	7.610×10^{-1}	2.656×10^{-1}		
Spaces Vol/Bone Vol.	2.256×10^{-1}	2.609×10^{-1}	2.376×10^{-1}	2.414×10^{-1}	1.795×10^{-2}
Spaces S.A.	7.604×10^{1}	4.531×10^{1}	1.650×10^{1}		
Spaces S.A/Vol.	6.951×10^{1}	5.954×10^{1}	6.212×10^{1}	6.372×10^{1}	5.175
O.S.A.T.	7.222×10^{-1}	6.987×10^{-1}	1.008×10^{-1}		
O.S.A.T/C.P.T.	6.437×10^{-3}	5.982×10^{-3}	9.081×10^{-4}	4.442×10^{-3}	3.069×10^{-3}
O.B.S.A.T.	1.596	4.050×10^{-1}	0.000		
O.B.S.A.T/O.S.A.T.	2.210	5.796×10^{-1}	0.000	9.299×10^{-1}	1.146
O.B.S.A.T/C.P.T.	1.422×10^{-2}	3.467×10^{-3}	0.000	5.896×10^{-3}	7.415×10^{-3}
R.B.S.A.T.	1.964×10^{-3}	4.290×10^{-1}	0.000		
R.B.S.A.T/C.P.T.	1.750×10^{-5}	3.673×10^{-3}	0.000	1.230×10^{-3}	2.115×10^{-3}

TWENTY-FIVE DAYS	91	.92	64	MEAN	STD. DEV.
C.P.T.	1.355×10^{2}	1.563×10^{2}	2.521×10^{2}	1.813×10^{2}	6.219×10^{1}
F.B.S.A.T.	5.399×10^{-1}	3.496	9.398		
F.B.S.A.T/C.P.T.	3.985×10^{-3}	2.237×10^{-2}	3.728×10^{-2}	2.121×10^{-2}	1.668×10^{-2}
Spaces Vol.	1.477×10^{-1}	1.446	2.173		
Spaces Vol/Bone Vol.	2.736×10^{-1}	4.136×10^{-1}	2.312×10^{-1}	3.061×10^{-1}	9.545×10^{-2}
Spaces S.A.	7.934	7.202×10^{1}	1.541×10^{2}		
Spaces S.A/Vol.	5.372×10^{1}	4.981×10^{1}	5.209×10^{1}	5.187×10^{1}	1.964
O.S.A.T.	2.386×10^{-1}	4.028×10^{-1}	1.665		
O.S.A.T/C.P.T.	1.761×10^{-3}	2.577×10^{-3}	6.604×10^{-3}	3.647×10^{-3}	2.593×10^{-3}
O.B.S.A.T.	0.000	2.691×10^{-1}	4.513		
O.B.S.A.T/O.S.A.T.	0.000	6.681×10^{-1}	2.710	1.126	1.412
O.B.S.A.T/C.P.T.	0.000	1.722×10^{-3}	1.790×10^{-2}	6.541×10^{-3}	9.873×10^{-3}
R.B.S.A.T.	4.345×10^{-2}	3.600×10^{-2}	1.289×10^{-1}		
R.B.S.A.T/C.P.T.	3.206×10^{-4}	2.303×10^{-4}	5.113×10^{-4}	3.540×10^{-4}	1.430×10^{-4}

THIRTY DAYS	65	. 90	81	MEAN	STD. DEV.
C.P.T.	2.403×10^{2}	1.516×10^{2}	1.259×10^{2}	1.726×10^{2}	6.002×10^{1}
F.B.S.A.T.	1.236×10^{1}	7.601	1.062×10^{1}		
F.B.S.A.T/C.P.T.	5.144×10^{-2}	5.020×10^{-2}	8.432×10^{-2}	6.199×10^{-2}	1.935×10^{-2}
Spaces Vol.	4.018	2.486	4.168		
Spaces Vol/Bone Vol.	3.251×10^{-1}	3.271×10^{-1}	3.925×10^{-1}	3.482×10^{-1}	3.835×10^{-2}
Spaces S.A.	1.839×10^{2}	1.184×10^{2}	1.560×10^{2}		
Spaces S.A/Vol.	4.577×10^{1}	4.763×10^{1}	3.743×10^{1}	4.361×10^{1}	5.432
O.S.A.T.	4.870×10^{-1}	5.424×10^{-1}	1.139		
O.S.A.T/C.P.T.	2.027×10^{-3}	3.578×10^{-3}	9.047×10^{-3}	4.884×10^{-3}	3.688×10^{-3}
O.B.S.A.T.	1.315×10^{-1}	1.189×10^{-1}	8.280×10^{-1}		
O.B.S.A.T/O.S.A.T.	2.700×10^{-1}	2.192×10^{-1}	7.269×10^{-1}	4.054×10^{-1}	2.796×10^{-1}
O.B.S.A.T/C.P.T.	5.472×10^{-4}	7.843×10^{-4}	6.576×10^{-4}	6.630×10^{-3}	1.183×10^{-4}
R.B.S.A.T.	7.052×10^{-2}	4.459×10^{-1}	7.601×10^{-3}		
R.B.S.A.T/C.P.T.	2.934×10^{-4}	2.941×10^{-3}	6.030×10^{-5}	1.098×10^{-3}	1.600×10^{-3}

FORTY DAYS	79	82	68	MEAN	STD. DEV.
C.P.T.	1.918×10^{2}	1.233×10^{2}	3.087×10^{2}	2.079×10^{2}	9.374×10^{1}
F.B.S.A.T.	7.094	2.165	2.602×10^{1}		
F.B.S.A.T/C.P.T.	3.698×10^{-2}	1.756×10^{-2}	8.430×10^{-2}	4.628×10^{-2}	3.433×10^{-2}
Spaces Vol.	2.173	6.834×10^{-1}	9.082		
Spaces Vol/Bone Vol.	3.063×10^{-1}	3.156×10^{-1}	3.490×10^{-1}	3.236×10^{-1}	2.245×10^{-2}
Spaces S.A.	9.926×10^{1}	2.849×10^{1}	3.418×10^{2}		
Spaces S.A/Vol.	4.567×10^{1}	4.169×10^{1}	3.763×10^{1}	4.166×10^{1}	4.020
O.S.A.T.	5.526×10^{-1}	5.310×10^{-1}	6.837×10^{-1}		
O.S.A.T/C.P.T.	2.881×10^{-3}	4.306×10^{-3}	2.215×10^{-3}	3.134×10^{-3}	1.068×10^{-3}
O.B.S.A.T.	2.098×10^{-1}	1.333×10^{-1}	5.924×10^{-1}		
O.B.S.A.T/O.S.A.T.	3.796×10^{-1}	2.510×10^{-1}	8.665×10^{-1}	4.990×10^{-1}	3.247×10^{-1}
O.B.S.A.T/C.P.T.	1.094×10^{-3}	1.081×10^{-3}	1.919×10^{-3}	1.365×10^{-3}	4.800×10^{-4}
R.B.S.A.T.	2.131×10^{-1}	2.875×10^{-1}	2.066×10^{-1}		
R.B.S.A.T/C.P.T.	1.111×10^{-3}	2.332×10^{-3}	6.692×10^{-4}	1.371×10^{-3}	8.613×10^{-4}

ONE HUNDRED DAYS	127	128	130	MEAN	STD. DEV.
C.P.T.	3.292×10^{2}	2.162×10^{2}	2.141×10^{2}	2.532×10^{2}	6.585×10^{1}
F.B.S.A.T.	9.410	5.409	8.181		
F.B.S.A.T/C.P.T.	2.858×10^{-2}	2.502×10^{-2}	3.821×10^{-2}	3.060×10^{-2}	6.823×10^{-3}
Spaces Vol.	3.343	1.948	1.729		
Spaces Vol/Bone Vol.	3.553×10^{-1}	3.601×10^{-1}	2.113×10^{-1}	3.089×10^{-1}	8.460×10^{-2}
Spaces S.A.	9.113×10^{1}	4.138×10^{1}	7.017×10^{1}		
Spaces S.A/Vol.	2.726×10^{1}	2.124×10^{1}	4.058×10^{1}	2.969×10^{1}	9.897
O.S.A.T.	1.975×10^{-2}	2.300×10^{-1}	4.303×10^{-1}		
O.S.A.T/C.P.T.	5.999×10^{-5}	1.064×10^{-3}	2.010×10^{-3}	1.045×10^{-3}	9.751×10^{-4}
O.B.S.A.T.	1.718×10^{-2}	6.522×10^{-3}	2.235×10^{-1}		
O.B.S.A.T/O.S.A.T.	8.699×10^{-1}	2.836×10^{-2}	5.194×10^{-1}	4.725×10^{-1}	4.227×10^{-1}
O.B.S.A.T/C.P.T.	5.210×10^{-5}	3.010×10^{-5}	1.044×10^{-3}	3.754×10^{-4}	5.790×10^{-4}
R.B.S.A.T.	1.038×10^{1}	4.089×10^{-1}	4.528×10^{-1}		
R.B.S.A.T/C.P.T.	3.153×10^{-4}	1.891×10^{-3}	2.115×10^{-3}	1.440×10^{-3}	9.807×10^{-4}

ONE HUNDRED AND EIGHTY DAYS	134	135	136	MEAN	STD. DEV.
C.P.T.	3.632×10^{2}	4.450×10^{2}	4.327×10^{2}	4.136×10^{2}	4.408×10^{1}
F.B.S.A.T	8.335	1.820×10^{1}	8.546		
F.B.S.A.T/C.P.T.	2.294×10^{-2}	4.090×10^{-2}	1.975×10^{-2}	2.786×10^{-2}	1.140×10^{-2}
Spaces Vol.	1.292	7.974	4.377		
Spaces Vol/Bone Vol.	1.550×10^{-1}	4.381×10^{-1}	5.122×10^{-1}	3.684×10^{-1}	1.885×10^{-1}
Spaces S.A.	5.299×10^{1}	1.198×10^{2}	5.633×10^{1}		
Spaces S.A/Vol.	4.101×10^{1}	1.502×10^{1}	1.287×10^{1}	2.297×10^{1}	1.566×10^{1}
O.S.A.T.	2.664×10^{-1}	1.398×10^{-1}	7.243×10^{-2}		
O.S.A.T/C.P.T.	7.330×10^{-4}	3.140×10^{-4}	1.670×10^{-4}	4.047×10^{-4}	2.934×10^{-4}
O.B.S.A.T.	0.000	8.100×10^{-2}	2.092×10^{-2}		
O.B.S.A.T/O.S.A.T.	0.000	5.794×10^{-1}	2.888×10^{-1}	2.894×10^{-1}	2.897×10^{-1}
O.B.S.A.T/C.P.T.	0.000	1.820×10^{-4}	4.830×10^{-5}	7.677×10^{-5}	9.381×10^{-5}
R.B.S.A.T.	1.895×10^{-1}	6.578×10^{-2}	2.908×10^{-1}		
R.B.S.A.T/C.P.T.	5.210×10^{-4}	1.470×10^{-4}	6.720×10^{-4}	4.467×10^{-4}	2.700×10^{-4}

TABLE B

CYST FLOOR MAP MEASUREMENTS

EIGHT DAYS +	99	69	74	MEAN	STD. DEV.
C.F.A.	2.301×10^{1}	1.345×10^{1}	2.353×10^{1}	1.996×10^{1}	5.675
B.F.A.	0.000	0.000	0.000		
B.F.A./C.F.A.	0.000	0.000	0.000		
C.F.A.*	2.217×10^{1}	1.447×10^{1}	N.M.		
S.F.A.*/C.F.A.*	9.020×10^{-3}	2.027×10^{-1}	0.000	7.057×10^{-2}	1.145×10^{-1}
O.F.A.*/C.F.A.*	0.000	0.000	0.000		
N.F.A.*/C.F.A.*	9.910×10^{-1}	7.788×10^{-1}	1.000	9.233×10^{-1}	1.251×10^{-1}

\+ No smooth muscle prior to eight days; no bone prior to ten days

* Van Gieson stained sections only

N.M. Not measured

TEN DAYS	87	75 .	59	MEAN	STD. DEV.
C.F.A.	1.963×10^{1}	2.403×10^{1}	1.402×10^{1}	1.923×10^{1}	5.017
B.F.A.	1.350	1.033	2.666×10^{-1}		
B.F.A/C.F.A.	6.880×10^{-2}	4.298×10^{-2}	1.900×10^{-2}	4.359×10^{-2}	2.469×10^{-2}
M.T.B.	2.733×10^{-2}	4.010×10^{-2}	4.632×10^{-3}	2.402×10^{-2}	1.788×10^{-2}
C.F.A*	1.877×10^{1}	2.417×10^{1}	N.M.		
S.F.A*	1.533	6.433	0.000		
S.F.A*/C.F.A*	8.167×10^{-2}	2.661×10^{-1}	0.000	1.159×10^{-1}	1.362×10^{-1}
O.F.A*/C.F.A*	0.000	0.000	0.000		
N.F.A*/C.F.A*	8.188×10^{-1}	6.798×10^{-1}	9.810×10^{-1} **	8.265×10^{-1}	1.507×10^{-1}

** Neither A/Floor A. (Haemotoxylin and Eosin stained sections included)

* Van Gieson stained sections only

N.M. Not measured

TWELVE DAYS	88	60	76	MEAN	STD. DEV.
C.F.A.	1.712×10^{1}	1.462×10^{1}	2.075×10^{1}	1.750×10^{1}	3.082
B.F.A.	3.567	3.783	1.817		
B.F.A/C.F.A.	2.084×10^{-1}	2.558×10^{-1}	8.755×10^{-2}	1.849×10^{-1}	8.880×10^{-2}
M.T.B.	5.360×10^{-2}	4.297×10^{-2}	5.725×10^{-2}	5.130×10^{-2}	7.245×10^{-3}
C.F.A.	1.807×10^{1}	1.480×10^{1}	2.070×10^{1}		
S.F.A*	4.818	2.167	9.333		
S.F.A*/C.F.A*	2.666×10^{-1}	1.464×10^{-1}	4.509×10^{-1}	2.880×10^{-1}	1.533×10^{-1}
O.F.A*/C.F.A*	0.000	0.000	0.000		
N.F.A*/C.F.A*	5.156×10^{-1}	6.441×10^{-1}	4.477×10^{-1}	5.358×10^{-1}	9.969×10^{-2}

* Van Gieson stained sections only

FOURTEEN DAYS	77	89 .	53	MEAN	STD. DEV.
C.F.A.	1.012×10^{1}	1.788×10^{1}	3.812×10^{1}	2.204×10^{1}	1.446×10^{1}
B.F.A.	2.900	5.133	1.398×10^{1}		
B.F.A/C.F.A	2.866×10^{-1}	2.871×10^{-1}	3.667×10^{-1}	3.135×10^{-1}	4.593×10^{-2}
M.T.B.	8.270×10^{-2}	2.385×10^{-2}	1.343×10^{-1}	8.030×10^{-2}	5.520×10^{-2}
C.F.A*	1.070×10^{1}	1.857×10^{1}	3.697×10^{1}		
S.F.A*	2.733	1.333	9.000		
S.F.A*/C.F.A*	2.554×10^{-1}	7.178×10^{-2}	2.434×10^{-1}	1.902×10^{-1}	1.027×10^{-1}
O.F.A*/C.F.A*	6.542×10^{-2}	0.000	0.000	2.180×10^{-2}	3.768×10^{-2}
N.F.A*/C.F.A*	5.483×10^{-1}	6.607×10^{-1}	3.427×10^{-1}	5.172×10^{-1}	1.612×10^{-1}

* Van Gieson stained sections only

SEVENTEEN DAYS	101	108	95	MEAN	STD. DEV.
C.F.A.	1.082×10^{1}	3.437×10^{1}	1.902×10^{1}	2.140×10^{1}	1.195×10^{1}
B.F.A	2.633	2.208×10^{1}	5.316		
B.F.A/C.F.A.	2.434×10^{-1}	6.424×10^{-1}	2.795×10^{-1}	3.884×10^{-1}	2.206×10^{-1}
M.T.B.	9.030×10^{-2}	8.250×10^{-2}	1.144×10^{-1}	9.570×10^{-2}	1.650×10^{-2}
C.F.A*	1.040×10^{1}	3.393×10^{1}	2.023×10^{1}		
S.F.A*	5.266	5.433	8.800		
S.F.A*/C.F.A*	5.063×10^{-1}	1.601×10^{-1}	4.350×10^{-1}	3.674×10^{-1}	1.823×10^{-1}
O.F.A*/C.F.A*	9.610×10^{-3}	4.420×10^{-2}	0.000	1.793×10^{-2}	2.302×10^{-2}
N.F.A/C.F.A*	2.215×10^{-1}	2.299×10^{-1}	2.966×10^{-1}	2.493×10^{-1}	4.110×10^{-2}

* Van Gieson stained sections only

TWENTY DAYS	94	93 .	63	MEAN	STD. DEV.
C.F.A.	1.971×10^{1}	1.918×10^{1}	2.082×10^{1}	1.990×10^{1}	8.360×10^{-1}
B.F.A.	3.483	1.325×10^{1}	7.250		
B.F.A/C.F.A.	1.767×10^{-1}	6.908×10^{-1}	3.482×10^{-1}	4.019×10^{-1}	2.661×10^{-1}
M.T.B.	8.020×10^{-2}	8.810×10^{-2}	9.850×10^{-2}	8.900×10^{-2}	9.080×10^{-3}
C.F.A*	1.770×10^{1}	1.937×10^{1}	2.130×10^{1}		
S.F.A*	1.169×10^{1}	1.133	5.800		
S.F.A*/C.F.A*	6.604×10^{-1}	5.849×10^{-2}	2.723×10^{-1}	3.304×10^{-1}	3.051×10^{-1}
O.F.A*/C.F.A*	0.000	0.000	0.000		
N.F.A*/C.F.A*	3.107×10^{-1}	2.048×10^{-1}	3.975×10^{-1}	3.043×10^{-1}	9.643×10^{-2}

* Van Gieson stained sections only

TWENTY-FIVE DAYS	92	64	91	MEAN	STD. DEV.
C.F.A.	2.642×10^{1}	5.370×10^{1}	2.170×10^{1}	3.394×10^{1}	1.727×10^{1}
B.F.A.	8.900	3.078×10^{1}	1.633		
B.F.A/C.F.A.	3.369×10^{-1}	5.732×10^{-1}	7.525×10^{-2}	3.284×10^{-1}	2.480×10^{-1}
M.T.B.	9.740×10^{-2}	7.610×10^{-2}	8.075×10^{-2}	8.475×10^{-2}	1.106×10^{-2}
C.F.A*	2.683×10^{1}	5.390×10^{1}	2.263×10^{1}		
S.F.A*	1.047×10^{1}	8.700	1.277×10^{1}		
S.F.A*/C.F.A*	3.902×10^{-1}	1.614×10^{-1}	5.643×10^{-1}	3.720×10^{-1}	2.020×10^{-1}
O.F.A*/C.F.A*	0.000	0.000	0.000		
N.F.A*/C.F.A*	2.696×10^{-1}	2.640×10^{-1}	3.800×10^{-1}	3.045×10^{-1}	6.534×10^{-2}

* Van Gieson stained sections only

THIRTY DAYS	81	65 .	90	MEAN	STD. DEV.
C.F.A.	2.185×10^{1}	4.287×10^{1}	2.300×10^{1}	2.924×10^{1}	1.182×10^{1}
B.F.A.	1.453×10^{1}	2.707×10^{1}	1.137×10^{1}		
B.F.A/C.F.A.	6.650×10^{-1}	6.314×10^{-1}	4.943×10^{-1}	5.969×10^{-1}	9.038×10^{-2}
M.T.B.	1.669×10^{-1}	1.141×10^{-1}	4.212×10^{-2}	1.077×10^{-1}	6.260×10^{-2}
C.F.A*	2.147×10^{1}	4.330×10^{1}	2.257×10^{1}		
S.F.A*	3.800	6.167	8.167		
S.F.A*/C.F.A*	1.770×10^{-1}	1.424×10^{-1}	3.618×10^{-1}	2.271×10^{-1}	1.179×10^{-1}
O.F.A/C.F.A*	2.328×10^{-2}	0.000	6.499×10^{-2}	2.942×10^{-2}	3.286×10^{-1}
N.F.A*/C.F.A*	2.484×10^{-1}	2.248×10^{-1}	2.053×10^{-1}	2.262×10^{-1}	2.121×10^{-2}

*Van Gieson stained sections only

FORTY DAYS	68	74	82	MEAN	STD. DEV.
C.F.A.	5.302×10^{1}	3.538×10^{1}	1.882×10^{1}	3.574×10^{1}	1.710×10^{1}
B.F.A.	3.340×10^{1}	1.797×10^{1}	3.717		
B.F.A/C.F.A.	6.299×10^{-1}	5.079×10^{-1}	1.975×10^{-1}	4.451×10^{-1}	2.229×10^{-1}
M.T.B.	1.962×10^{-1}	9.495×10^{-2}	1.336×10^{-1}	1.416×10^{-1}	5.110×10^{-2}
C.F.A*	5.300×10^{1}	3.270×10^{1}	1.990×10^{1}		
S.F.A*	1.777×10^{1}	1.290×10^{1}	1.450×10^{1}		
S.F.A*/C.F.A*	3.353×10^{-1}	3.945×10^{-1}	7.286×10^{-1}	4.861×10^{-1}	2.120×10^{-1}
O.F.A*/C.F.A*	3.770×10^{-3}	0.000	0.000	1.260×10^{-3}	2.170×10^{-3}
N.F.A*/C.F.A*	2.264×10^{-2}	1.641×10^{-1}	7.201×10^{-2}	8.625×10^{-2}	7.179×10^{-2}

* Van Gieson stained sections only

ONE HUNDRED DAYS	127	128	130	MEAN	STD. DEV.
C.F.A.	5.512×10^{1}	4.217×10^{1}	3.888×10^{1}	4.539×10^{1}	8.585
B.F.A.	1.935×10^{1}	9.517	2.130×10^{1}		
B.F.A/C.F.A.	3.510×10^{-1}	2.257×10^{-1}	5.478×10^{-1}	3.748×10^{-1}	1.624×10^{-1}
M.T.B.	2.431×10^{-1}	2.341×10^{-1}	1.872×10^{-1}	2.381×10^{-1}	4.863×10^{-2}
C.F.A*	6.597×10^{1}	4.397×10^{1}	3.947×10^{1}		
S.F.A*	3.117×10^{1}	3.707×10^{1}	1.343×10^{1}		
S.F.A*/C.F.A*	4.725×10^{-1}	8.431×10^{-1}	3.403×10^{-1}	5.520×10^{-1}	2.606×10^{-1}
O.F.A*/C.F.A*	0.000	9.097×10^{-2}	0.000	3.032×10^{-2}	5.252×10^{-2}
N.F.A*/C.F.A*	7.023×10^{-2}	6.670×10^{-2}	1.132×10^{-1}	8.338×10^{-2}	2.586×10^{-2}

* Van Gieson stained sections only

ONE HUNDRED AND EIGHTY DAYS	134	135	136	MEAN	STD. DEV.
C.F.A.	5.687×10^{1}	7.098×10^{1}	7.980×10^{1}	6.922×10^{1}	1.157×10^{1}
B.F.A.	1.700×10^{1}	2.135×10^{1}	8.083		
B.F.A/C.F.A.	2.989×10^{-1}	3.008×10^{-1}	1.013×10^{-1}	2.337×10^{-1}	1.146×10^{-1}
M.T.B.	2.451×10^{-1}	4.178×10^{-1}	5.075×10^{-1}	3.901×10^{-1}	1.333×10^{-1}
C.F.A*	5.577×10^{1}	7.213×10^{1}	7.823×10^{1}		
S.F.A*	3.890×10^{1}	6.073×10^{1}	6.850×10^{1}		
S.F.A*/C.F.A*	6.975×10^{1}	8.419×10^{-1}	8.756×10^{-1}	8.050×10^{-1}	9.460×10^{-2}
O.F.A*/C.F.A*	2.630×10^{-2}	0.000	4.260×10^{-2}	2.297×10^{-2}	2.147×10^{-2}
N.F.A*/C.F.A*	1.255×10^{-2}	4.621×10^{-2}	3.409×10^{-2}	3.095×10^{-2}	1.703×10^{-2}

* Van Gieson stained sections only

TABLE C

GRAPH DATA

FIG. NO.	TITLE	DAYS	SLOPE	INTERCEPT	n	t	p
48	C.P.T.	0-10	-1.936×10	2.844×10^{2}	18	-5.90	$<0.1\%$
	C.P.T.	10-25	3.441	7.314×10	18	1.65	N.S.
	C.P.T.	25-180	1.474	1.352×10^{2}	15	5.48	$<0.1\%$
144	C.P.T.	10-180	1.684	1.070×10^{2}	30	9.08	$<0.1\%$
108	F.B.S.A.T./C.P.T.	10-17	3.543×10^{-3}	-3.570×10^{-2}	12	3.98	$<0.5\%$
	F.B.S.A.T./C.P.T.	17-25	-5.511×10^{-4}	3.557×10^{2}	9	-0.36	N.S.
145	F.B.S.A.T./C.P.T.	25-180	-1.022×10^{-4}	4.525×10^{-2}	15	-1.03	N.S.
145	F.B.S.A.T./C.P.T.	30-180	-1.963×10^{-4}	5.886×10^{-2}	12	-2.02	N.S.
	Sp. Vol./Bone Vol.	12-25	5.160×10^{-3}	1.648×10^{-1}	15	1.58	N.S.
	Sp. Vol./Bone Vol.	25-180	2.358×10^{-4}	3.134×10^{-1}	15	0.58	N.S.
147	Sp. Vol./Bone Vol.	12-180	6.250×10^{-4}	2.615×10^{-1}	27	2.13	$<5.0\%$
109	Sp. S.A./Sp. Vol.	12-25	-1.841	9.869×10	15	-2.40	$<5.0\%$
148	Sp. S.A./Sp. Vol.	25-180	-1.644×10^{-1}	5.029×10	15	-4.34	$<0.1\%$
	Sp. S.A./Sp. Vol.	12-180	-2.823×10^{-1}	6.589×10	27	-5.08	$<0.1\%$

FIG. NO.	TITLE	DAYS	SLOPE	INTERCEPT	n	t	p
49	O.S.A.T./C.P.T.	2-10	1.479×10^{-3}	-3.275×10^{-3}	15	4.97	$<$0.1%
110	O.S.A.T./C.P.T.	10-25	-4.693×10^{-4}	1.365×10^{-2}	18	-2.49	2.5%
149	O.S.A.T./C.P.T.	25-180	-2.547×10^{-5}	4.533×10^{-3}	15	-2.89	$<$2.0%
	O.S.A.T./C.P.T.	10-180	-4.022×10^{-5}	6.339×10^{-3}	30	-3.02	$<$1.0%
111	O.B.S.A.T./O.S.A.T.	10-25	8.222×10^{2}	-8.435×10^{-1}	18	2.57	$<$2.5%
	O.B.S.A.T./O.S.A.T.	25-180	-2.864×10^{-3}	7.731×10^{-1}	15	-0.98	N.S.
50	E.F.A./C.F.A.	2-8	1.478×10^{-1}	-2.982×10^{-1}	12	7.68	$<$0.1%
	C.F.A.	10-25	8.633×10^{-1}	8.234	18	1.88	N.S.
	C.F.A.	25-180	2.377×10^{-1}	2.487×10^{1}	15	4.41	$<$0.1%
150	C.F.A.	10-180	2.849×10^{-1}	1.859×10^{1}	30	7.62	$<$0.1%
112	B.F.A./C.F.A.	10-17	4.935×10^{-2}	4.215×10^{-1}	12	3.89	0.5%
	B.F.A./C.F.A.	17-25	-8.545×10^{-3}	5.546×10^{-1}	9	-0.37	N.S.
	B.F.A./C.F.A.	25-180	-1.434×10^{-3}	5.034×10^{-1}	15	-1.79	N.S.
151	B.F.A./C.F.A.	30-180	-2.043×10^{-3}	5.914×10^{-1}	12	-2.84	2.0%

FIG. NO.	TITLE	DAYS	SLOPE	INTERCEPT	n	t	p
113	M.T.B.	8-17	1.108×10^{-2}	-8.497×10^{-2}	15	5.41	$<$0.1%
	M.T.B.	17-25	-1.318×10^{-3}	1.170×10^{-1}	9	-1.10	N.S.
152	M.T.B.	25-180	1.889×10^{-3}	5.074×10^{-2}	15	6.64	$<$0.1%
115	S.F.A.*/C.F.A.*	6-25	1.997×10^{-2}	6.286×10^{-2}	24	3.68	$<$0.2%
153	S.F.A.*/C.F.A.*	25-180	3.017×10^{-3}	2.622×10^{-1}	15	3.76	0.2%
116	N.F.A.*/C.F.A.*	6-25	-4.193×10^{-2}	1.170	24	-7.86	$<$0.1%
154	N.F.A.*/C.F.A.*	25-180	-1.327×10^{-3}	2.458×10^{-1}	15	-3.71	0.2%

* Van Gieson stained sections only

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